An investigation into potential applications of spray-dried microparticles for use in the field of gene delivery

Mushtaq, Yasmin

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An Investigation into Potential Applications of Spray-Dried Microparticles for Use in the Field of Gene Delivery

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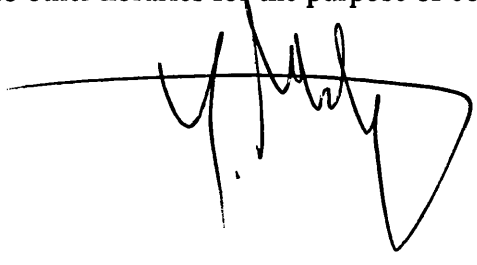

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Summary

Biodegradable microparticles are already a well-established form of drug delivery. They can be used to deliver peptides, proteins or low molecular weight drugs by various routes for administration e.g. parenteral, pulmonary, oral and nasal. Currently their potential for use in the field of gene delivery is being explored.

This study focused on utilising Quadrant Healthcare's proprietary spray drying technique to produce two different types of microparticles. Firstly, soluble mannitol microparticles with entrapped DNA/lipid complexes, which have potential for delivery of DNA to the lung. Secondly, cross-linked defatted human serum albumin microparticles which may have use as carriers for DNA vaccines for delivery to phagocytic macrophages or professional antigen presenting cells.

The mannitol microparticles were physically characterised through microscopy and particle size and found to be of appropriate size for lung deposition. *In vitro* gene expression experiments were used to assess the biological activity of the spray-dried DNA. DNA appeared to survive spray drying but was unstable on storage. The initial gene expression experiments were not suitably sensitive and suffered interference perhaps due to endogenous galactosidases. An alternative gene expression assay was established utilising the luciferase reporter gene. Luciferase expression was detectable after 4 hours and peaked 24-32 hours after transfection.

Cross-linked albumin microparticles were physically characterised via SEM and particle size. The microparticles were evaluated for their loading capacity for DNA with aid of cationic lipids, alky trimethyl bromides and polycations, polylysine and protamine. *In vitro* transfection experiments suggested that the particles were unable to transfect non-phagocytic cells. Phagocytic cells took up the particles *in vitro*. Animal experiments focused on assessing whether the presence of microparticles *in vivo* affected the number of macrophages present in RIF-1 tumour models. Macrophage presence was quantified in tumour tissue with the aid of enzyme histochemistry. An immune response was mediated in response to the presence of microparticles *in vivo*. Attempts were also made to study the fate of the particles after intratumoral injection using fluorescently labelled microparticles. Microparticles were located near the site of injection and in the periphery of the tumour tissue.

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Abbreviations

APC	antigen presenting cells
αMSH	alpha melanocyte stimulating hormone
ß-gal	ß-galactosidase
BSA	bovine serum albumin
bp	base pairs
CDTA	1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid
CMV	cytomegalovirus
CpG	cytosine phosphate guanine
CTAB	alkyl trimethylammonium bromides
DABCO	diazabicyclo[2,2,2,]octane
DC	dendritic cells
DHSA	defatted human serum albumin
DMSO	dimethylsulphoxide
DOPE	dioleoylphosphatidylethanolamine
DOTAP	N-[-1-(2,3-dioleolyoxy)propyl]-N,N,N-trimethylammonium-methylsulphate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDAC	1-Ethyl-3-(3-dimethylaminopropyl)carbodimide Hydrochloride
EDTA	ethylene diamine tetra-acetic acid
FCS	foetal calf serum
FITC	fluoresceinisothiocyanate
g	gramme (s)
GDV	gene delivery vector
HBS	Hepes-buffered saline
HCL	hydrochloric acid
HEPES	*N*-(2-hydroxyethyl)piperazine-N-2'-ethane sulphonic acid
HPLC	high-performance liquid chromatography
HSA	human serum albumin
IL	interleukin
IMS	industrial methylated spirit
IFN	interferon

lacZ	ß-galactosidase
LB	Luria broth
luc	luciferase gene
µ	micro
m	milli
M	molar
MEM	Eagle's minimum essential medium
MHC	major histocompatibility complex
MPE	microparticle/protamine/EDAC
ONPG	σ-nitrophenyl- ß-D-galactoside
PBS	phosphate buffered saline
pLL	polylysine
RIF	radiation-induced fibrosarcoma
RLU	relative luminescence units
RSV	rous sarcoma virus
SE	standard error
SEM	standard error of the mean
TNF	tumour necrosis factor
TOTO	thiazole orange dimer
Tris	Tris(hydroxymethyl)aminomethane
U	units
UV	ultraviolet
VMD	volume median diameter
v/v	volume by volume
w/v	weight by volume
w/w	weight by weight
YOYO-1	oxazole yellow dimer

CHAPTER 1

INTRODUCTION

Somatic gene therapy is a novel therapeutic approach defined as the introduction of genetic information into cells to achieve production of beneficial proteins to correct or modulate a disease. Somatic gene therapy can target many severe or debilitating diseases, including inherited genetic disorders such as cystic fibrosis and haemophilia, as well as acquired, multifactorial diseases such as arthritis, AIDS and cancers (Miller, 1992; Anderson, 1992; Friedmann *et al.*, 1989; Michael and Curiel, 1994; Ledley, 1994a, 1994b). The intended purpose of gene therapy products is to overcome some of the limitations associated with the clinical use of protein drugs, including low bioavailability, poor stability and pharmacokinetics and high cost of manufacture. Gene therapy has great potential as it offers a new range of therapies based on selective, regulated expression of endogenous proteins or exogenous vaccines, at the appropriate target site. The initial approaches to gene therapy have exhibited promising pharmacological effects in several animal models of genetic and acquired diseases. Clinical trials have demonstrated that genes can be introduced into patients by several different methods and will express potentially therapeutic gene products (Crystal *et al.*, 1993; Caplen *et al.*, 1995; Zabner *et al.*, 1993; Grossman *et al* 1995). However, from the published clinical data it is apparent that there is a lack of clinical efficacy as a result of low gene expression following administration of a gene medicine. The expression system is an essential component of activity, but the design and optimisation of the delivery vehicle will also be a major part of the development of a successful gene medicine. The gene delivery process can be seen as a series of hurdles (both extracellular and intracellular) that successively deplete the mass of DNA that

progresses towards the target site. Improvement in the transfer efficiency across any individual step would be expected to improve overall efficiency. Therefore along with gaining an understanding into the various hurdles or barriers to gene delivery, basic research and development into formulating therapeutic DNA into a pharmaceutical product is also needed towards the formulation of successful, and site-specific gene therapy technologies. There is good reason to believe that different biological targets and pathologies will require different gene therapy technologies.

1.1. BARRIERS TO GENE DELIVERY

The enormity of the task of gene delivery can be better appreciated if the biological barriers hindering gene transfer within the body are considered. The exact barriers are dependent on the gene therapy strategy sought, and particularly the route of administration of the genetic material in the body. Transgenes may be introduced either *ex vivo* or *in vivo*. The current advantage of *ex vivo* strategies is that many of the biological barriers encountered within the body can be avoided. This is achieved by the removal of the target tissue and *in vitro* transfection of the target cells, often followed by selection processes designed to enhance the proportion of transfected cells. Finally, the surviving transfected cells are reintroduced into the body. There are, however many obstacles inherent to *ex vivo* gene delivery approaches, mainly involving limitations to the efficiency of transgene expression *in vitro* and the time and labour intensive processes involved with selection, which generally result in significant tissue loss, and the reintroduction of transformed cells *in vivo* (Kabanov, 1999).

The situation becomes more complicated in the case of *in vivo*-based strategies. Practically every accepted *in vivo* route of administration has been considered for gene

delivery, including systemic, intramuscular, oral, topical, ocular and alveolar. At present the most significant obstacle hindering *in vivo* gene therapy is delivery of the gene to the target site, and at levels that are biologically relevant. In the case of systemic administration for example, the availability of non-viral vectors to the target site is decreased by the uptake of vectors by non-target tissues (Mahato *et al.*, 1997). DNA-containing complexes are generally too large to be easily taken up within the capillary tissues, and therefore become targets for elimination by cells of the reticuloendothelial system (RES), particularly in the liver. In addition to the physical barriers created by the formed body tissues, interactions with macromolecules present in the body fluids, such as serum proteins, can strongly affect the properties of the non-viral vector, perhaps modifying its tissue distribution (Kabanov, 1999).

In certain cases, following administration in the body the non-viral vector is separated from its target by a natural biological barrier. For instance, the blood-brain barrier impedes gene therapy targeting tumours in the central nervous system (review by Kabanov, 1999). Another example is the intestinal barrier, which has proven to be a major obstacle by decreasing the bioavailability of antisense oligonucleotides following oral administration (Agarwal *et al.*, 1995). Sometimes the target site itself provides a formidable barrier for gene delivery, e.g. low permeability of tumour vasculature can significantly complicate gene transfer in solid tumours (Lemmon *et al.*, 1997).

In addition to the physical barriers to gene therapy present within the body, degradation of vectors prior to reaching their targets must be avoided. Enzymatic digestion of the DNA by nucleases present in the body fluids can result in the elimination of the transgene before it reaches the target site. To avoid degradation non-viral vectors must remain stable until they reach their target. However, the non-viral

vectors cannot be too stable, because the DNA must be released during interaction with the target cell to enable the transgene expression.

The problems associated with transgene delivery do not end once the vector has arrived at the outer membrane of the target cells. The processes of transgene entry into, and movement through, the intracellular environment is currently the subject of increasing attention (Zabner *et al.*, 1995; Tseng *et al.*, 1997). Compared to a DNA molecule, a cell is an enormous place in which the DNA molecule can easily get lost. The transgene may be sequestered within the wrong compartments of the cell, leading to degradation instead of trafficking properly to its intracellular target. The transfection process can be divided into four major elements, the first process is the entry of DNA into the cell. The second involves the trafficking of the transgene through the cytoplasm, including both endosomal and lysosomal compartments. The third phase is the entry of DNA into the nucleus. The final element involves the ability to escape intranuclear mechanisms designed to purge foreign DNA, followed by the actual transcription of the transgene.

It is generally accepted that the first element, delivery of the non-viral vector into the cell, occurs through an endocytosis process, although the efficiency of this process is unknown (Zabner *et al.*, 1995; Tseng *et al.*, 1997; Wrobel and Collins, 1995). Subsequent intracellular vesicular trafficking involves targeting of vesicles to their correct destination, which is controlled by unique compartment-specific proteins (Slepnev and Camilli, 1998). The precise transport of naturally occurring molecules between intracellular organelles is crucial for normal cell functioning and viability. From this point of view, the non-viral vectors are 'alien' to the cell and they usually lack proper recognition characteristics necessary to direct their transport inside the cell. Furthermore, a common problem of non-viral gene delivery systems is how to

overcome transgene degradation within the lysosomal compartments and increase transgene release from the transport vesicles. The subsequent mechanisms involved in transgene expression are not well defined, but many believe that these elements are dependent upon cellular mitosis, evidenced by the observation that primary cells, in contrast to rapidly dividing, transformed cell lines, are typically refractory to transfection (Kabanov, 1999).

Overall, multiple barriers exist that impede gene transfer both *in vitro* and *in vivo*, and formidable strategies will be required to overcome these problems. There are two primary branches of research striving to overcome these problems. One involves the design of the transgene itself to help to achieve expression levels as well as tissue and developmental specificity of transgene expression. The second branch, which is the primary focus of this chapter, is the selection of efficient transgene strategies.

1.2. STRATEGIES FOR SOMATIC GENE DELIVERY

A number of methods for transferring genes to cells have been explored (Miller, 1992; Kay *et al.*, 1993; Ledley, 1995; Knowles *et al.*, 1995). Each of these methods offers distinct advantages as well as disadvantages. Methods can be distinguished based on the nature of the product to be administered to the patient. They include the use of: (1) cells, genetically modified *ex vivo* with viruses or other gene-transfer methods prior to re-introduction into the patients body, (2) viral vectors, based on genetically engineered, attentuated or defective viruses, (3) plasmid DNA alone, or formulated using synthetic delivery systems for direct *in vivo* administration.

1.2.1. Cell-Based *Ex Vivo* Gene Therapy

The first approved clinical trial involved an *ex vivo* approach (Blaese *et al.*, 1995). Retroviral vectors were used to introduce *ex vivo* the adenosine deaminase (ADA) gene permanently into the chromosomes of white blood cells from two patients suffering from severe combined immunodeficiency (SCID). These genetically engineered cells were then administered as an autologous transfusion. The biological benefits of this first *ex vivo* gene therapy trial have been relatively limited, partly because of the low number of cells containing the therapeutic gene and subsequent low levels of expression of ADA. An analogous approach has been used in a pilot study of gene therapy for familial hypercholesterolemia (LDL-receptor deficiency) (Grossman *et al*, 1995). Detectable expression levels of LDL-receptor were found in a small number of hepatic cells.

However, there are several factors that limit the clinical approach of *ex vivo* gene therapy the most important being the fact it is more invasive than a conventional pharmaceutical product. Also cultivation, genetic manipulation, quality control and transplantation of autologous cells is expensive relative to the cost of a conventional pharmaceutical or biological product as well as the fact it is more invasive than a conventional pharmaceutical product.

1.2.2. Viral-Based *In vivo* Gene Therapy

Viral-mediated gene therapy involves the genetic engineering of attenuated or defective viruses. These viral vectors are designed to transfer therapeutic genes into cells without causing viral disease, and function on the premise that they utilise highly

evolved pathways of infection to deliver and express genes in the body. Such pathways provide viral vectors with access to a number of target cells, uptake via receptor-mediated endocytosis and efficient intracellular trafficking from the endosomes to the nucleus. Due to their natural ability to infect cells, several viruses, such as retrovirus, adenovirus, adeno-associated virus have been investigated for *in vivo* gene delivery. These and more viral vectors have been extensively reviewed in the following articles, Miller, 1992; Takakura and Hashida, 1996; Roemer and Friedmann, 1992; Mulligan, 1993; Anderson 1998; Ledley, 1996. Viral vectors will only be reviewed briefly as they are not the main focus of this thesis.

Retroviruses currently account for 60% of the vectors types used in clinical trials. The Retroviral vectors are essentially enveloped RNA viruses which can carry out efficient gene transfer into many cell types and can stably integrate into the host cell genome, thereby providing possibility of long term expression. However, they can only infect replicating cells and as they integrate genes into the host genome permanently it removes the ability to modify or terminate the therapy in response to any adverse side effects. Alternatively Adenoviruses, are human DNA viruses, which can efficiently infect both dividing and non-dividing cells and do not integrate genes into the host genome. Also they are large and therefore can carry larger foreign DNA inserts (upto 35kb) where as the retrovirus can only carry less than 10 kb. Another human DNA virus is the Adeno-associated virus, it is essentially non-pathogenic and is the most widely found virus in the human population (about 80% of humans have antibodies against AAV). It is the only known mammalian virus that shows preferential integration into a specific region in the human genome (in the short arm of human chromosome 19). However, like the retrovirus it only has a small genome only allowing room for 4.8 kb of foreign DNA.

There are many more virus based vectors under investigation but the underlying factor for all these viral vectors are problems with their safety when introduced into the human genome, apart from their inefficiency to produce high levels of gene transfer and difficulty to manufacture in large quantities. Until safety of these vectors can be improved and guaranteed non-viral vectors will be the preferred choice by many scientists.

1.2.3. Plasmid-Based, Non-viral Gene Delivery

The non-viral gene delivery methods overcome many of the inherent safety issues associated with the use of viral vectors. Plasmids of high quality can be purified from bacterial fermentation in high yield. Chemical stability of plasmids can be maintained at or near ambient temperatures (Manning, 1989) and plasmids may be stored for long periods as aqueous suspensions or lyophilised material. A plasmid-based delivery system generally consists of three components: a) a gene encoding a therapeutic protein or reporter protein, b) a plasmid-based gene expression system that controls the function of a gene within the target cell, c) a synthetic delivery system that controls the location of the gene within the body.

Non-viral gene delivery methods can be classified as either physical, non-particulate and particulate systems. Physical methods include microinjection (Capecchi, 1980), electroporation (Winterbourne *et al.*, 1988) and particle bombardment (Yang *et al.*, 1990). The afore mentioned are regular laboratory techniques but are less suitable for clinical purposes because of toxicity and lack of practicality although electroporation from a needle tip or particle bombardment using a 'gene gun' may find

some use for local administration or vaccination strategies. Non-particulate and particulate systems will be the focus of the remainder of this review.

1.2.3.1. Non-particulate systems, Naked DNA

The simplest approach to non-viral delivery systems is the direct gene transfer with naked plasmid DNA. Plasmids are colloidal systems with characteristics unfavourable to cellular uptake (specific hydrodynamic size, very hydrophilic and have a net negative surface charge owing to the phosphate group on each nucleotide, and lability), despite this fact plasmid DNA is active *in vivo* under certain circumstances. Expression was first observed after injection of purified DNA into skeletal muscle (Wolff *et al.*, 1990, 1991, 1992a). Although initial studies demonstrated that expression of reporter genes lasted for several months, expression was generally at low levels during this time. These early results stimulated further refinement in plasmid DNA construction (Hartikka *et al.*, 1996) and delivery vehicles (Mumper *et al.*, 1996), both of which significantly enhanced the level of transgene expression.

Plasmid expression has subsequently been found in other organs including cardiac muscle (Lin *et al*, 1991), endothelial cells (Riessen *et al.*, 1993), thyroid gland (Sikes *et al*, 1994), liver (Hickman *et al.*, 1995; Budker *et al.*, 1996), lungs (Meyer *et al.*, 1995), tumour cells (Yang and Huang, 1996), and the brain (Schwartz *et al.*, 1995). The mechanism by which cells take up and express purified DNA remains puzzling. It has been proposed that, in muscle DNA is taken up through the T-tubules into the body of the cell (Wolff *et al.*, 1992b). In thyroid it has been proposed that DNA is taken up passively during the normal course of endocytosis by thyroid follicular cells (Sikes *et al.*, 1994).

1.2.3.2. Non-Condensing Interactive Polymers

The level of gene expression following intramuscular injection of unformulated plasmids into non-occluded muscle tissue remains insufficient for a number of applications, resulting in highly variable levels of expression. Following on from this formulations of plasmid DNA for intramuscular administration with polymers such as polyvinyl pyrridone (PVP) and polyvinyl alcohol (PVA) (reviewed by Ledley, 1996) have been explored as a means for enhancing stability, retention, and dispersion of DNA within muscle (Mumper *et al.*, 1995 Mumper *et al.*, 1996). PVA and PVP interact with plasmids through hydrogen bonding (Mumper *et al.*, 1998). This interaction affords the plasmid several properties, the plasmid gains some nuclease protection, through being provided with a hydrophobic coat by the polymers. This hydrophobicity also led to increase in uptake and movement within the muscle. A 10-fold enhancement in gene expression over naked DNA has been observed in rat muscle using the PVP and PVA polymer system (Mumper *et al.*, 1995; Mumper *et al.*, 1996). Also intramuscular injection of PVP-based plasmid formulations provide reproducible levels of gene expression along with a continued increase in expression as plasmid dose is increased to levels above 100 μg have been observed (Mumper *et al.*, 1996). The ability of PVP-based formulations to extend the dose response kinetics suggests that the plasmid DNA enters cells by a different pathway than naked DNA (Levy *et al.*, 1996), or that the PVP alters the capacity of one of the rate-limiting steps in this pathway.

1.2.3.3. Particulate Systems

Non-viral delivery systems are primarily designed to interact with the phosphate groups of nucleotides, reducing the net negative surface charge of plasmids. Neutralisation of more than 90% of the plasmids negative charges results in condensation of the plasmid into a particle. In neutralising the plasmids negative charges the molecule can interact with biological membranes which also have a net negative charge. Uptake of cationic particles takes place non-specifically by adsorptive endocytosis. Today the principle ways of accomplishing condensation and packaging of plasmid DNA are either with hydrophobic cations or hydrophilic polycations. Hydrophobic cations form liposomes or micelles that can interact with DNA and reorganise into a cationic lipid/DNA complex called a 'lipoplex', the process of DNA delivery mediated by cationic lipids is termed 'lipofection'. The hydrophilic 'polycations', mainly polymers also form complexes with DNA, such a complex is described as a 'polyplex' (Felgner *et al.*, 1997; Felgner, 1999) and cationic polymer mediated DNA delivery is termed 'polyfection'.

The following discussion on non-viral particulate systems will look, not only at the first generation systems, but also at the more sophisticated systems, which are evolving to gain some of the elegant functions and complexities of viruses. The incorporation of both viral and non-viral functional groups into a transfer system should allow such a system to surmount the various extra- and intra-cellular barriers to gene delivery. The expanding array of non-viral vector platforms will be selected ultimately on their ability to effect gene transfer *in vivo*.

The levels of gene expression obtainable in animals with a number of non-viral formulations are in some instances comparable to levels seen with viral gene delivery

systems. However, viruses are still much more efficient gene delivery systems. For example, adenoviruses are typically used *in vitro* at a multiplicity of infection of about 100. Under these conditions, in which each cell is exposed to an average of only 100 functional adenovirus particles, most of the cells exposed to the virus become transfected. To achieve comparable levels of transgene expression with non-viral systems, the cells must be exposed to about 1 million copies of plasmid per cell (5μg plasmid/million cells). Similar differences in efficiency are apparent from *in vivo* studies. In mouse lung, about 2×10^{13} copies of plasmid (130μg) were used, whereas, in a typical adenovirus vector study 10^9 virus particles are administered. There is therefore much scope for improving the efficiency of non-viral gene delivery and it is believed that much of the improvement will come from the development of new and improved non-viral gene delivery systems.

1.2.3.3.1. Cationic Lipid-Based Gene Delivery Systems

The most investigated approach in non-viral gene therapy are cationic lipid-based systems. *In vivo* they may enhance gene delivery in several ways including: (a) protecting DNA against degradation, (b) modifying the size, charge and surface characteristics of the DNA-containing particulate to control its biodistribution within the body and access to the target cell, (c) enhancing the interaction of DNA with the surface of the target cell, (d) inducing endocytosis, (e) enhancing release of DNA from the endosome and (f) enhancing the entry of DNA into the nucleus.

The prototype cationic lipid for gene transfer *in vitro* is Lipofectin a mixture of DOTMA and co-lipid DOPE (Felgner *et al.*, 1987, 1994). Since its successful introduction a number of cationic lipids have been designed and synthesised, the more

recent cationic lipids include the quaternary amine-based cationic lipids e.g. DMRIE and DOTAP (Felgner *et al.*, 1994; Liu *et al.*, 1995) and the cholesterol based cationic lipids e.g. DC-Chol (Goa and Huang, 1995). Neutral lipids such as DOPE and cholesterol are generally included in lipoplexes as colipids as they are thought to facilitate fusion between the lipids in the lipoplex and the endosomal membrane, destabilising the endosome and releasing the plasmids or lipoplexes into the body of the cell.

Differing structures have been reported on mixing cationic lipids with plasmid DNA. Gustafasson observed lipoplex systems that resembled multilamellar vesicles by cryoelectron microscopy suggesting that lipid bilayers were able to maintain structure and form stacks of bilayers with DNA intercalated between the bilayers (Gustafasson *et al.*, 1995). Gao and Huang have reported to observe heterologous structures they describe as 'spaghetti and meatball complexes' (Gao and Huang, 1995). Another proposed model involves the reorganisation of the initial lipid structure for instance cationic liposomes by fusion of adjacent lipid structures induced by the polyanionic plasmid (Gershon *et al.*, 1993; Sternberg *et al.*, 1994).

Due to the intensive research in this area, novel cationic lipid molecules are regularly reported and it has also been discovered that the optimal composition of the lipid-based systems required to achieve maximal *in vitro* gene expression varies from cell line to cell line however, despite all this research no definite structure-activity relationship has emerged between the structure of the charge groups or fatty acid component and gene transfer (Felgner *et al.*, 1994; Gao and Huang, 1995).

A small number of mechanistic studies have been initiated to try and characterise the pathway by which plasmid DNA enters the nucleus

Cationic lipids have been used successfully to deliver genes to several target tissues *in vivo*. The lung appears to be particularly susceptible to cationic lipid-mediated delivery. Various lipid formulations have successfully transfected pulmonary epithelial cells after instillation and nebulisation to the airways (Caplen *et al.*, 1995; Logan *et al.*, 1995; Middleton *et al.*, 1994; Sorcher *et al.*, 1994). Successful expression of α1-anti-trypsin (Canonico *et al.*, 1994a,b) and cystic fibrosis transmembrane conductance regulator (CFTR) (Yoshimura *et al.*, 1992; Alton *et al* 1993; Hyde *et al.*, 1993), within the lung in animal studies has led to clinical trials of gene therapy for genetic deficiencies of these proteins.

Several studies have also employed lipoplexes for administration directly into tumour masses for anticancer therapy. Initial studies were aimed at expressing a foreign antigen within tumour cells to enhance the immunogenicity of the tumour or presentation of tumour-specific antigens. Studies in mice demonstrated the feasibility of gene delivery and expression in tumour cells after direct injection as well as stimulation of a cellular immune response against the tumour (Plautz *et al.*, 1993; Conry *et al.*, 1994). A clinical trial has been reported in which the gene encoding HLA B7 was introduced into melanomas as a xenoantigen, with the expectation that the immune response against this antigen, as well as potentially enhanced presentation of tumour-specific antigens, would lead to immunologically mediated destruction of the injected tumour and potentially a tumour vaccine against systemic disease (reviewed by Ledley, 1995).

1.2.3.3.2. Polypeptide Gene Delivery

Formulations of DNA with protein ligands have been developed to achieve receptor-mediated uptake of plasmids into certain target cells. Proteins are commonly complexed with DNA by covalently coupling the protein to polylysine and binding this complex to DNA through ionic interaction between the positively charged polylysine and the negatively charged DNA (Wagner *et al.*, 1990; Wagner *et al.*, 1991a). The interaction of polylysine with DNA also condenses the DNA and toroidal protein/DNA structures as small as 80 nm diameter have been reported (Wagner *et al.*, 1991a). The protein component of the resulting complex retains its ability to interact specifically with cognate receptors on the target cell, which leads to internalisation of the DNA into the cell by receptor-mediated endocytosis. Several different protein ligands have been used effectively *in vitro*. For example transferrin/polylysine/DNA complexes have shown effective gene delivery into various cell types *in vitro* including hematopoietic cells, T cells and pulmonary epithelium cells (Wagner *et al.*, 1990; Zenke *et al.*, 1990; Buschle *et al.*, 1995; Harris *et al.*, 1993). Surfactant B/polylysine/DNA complexes and anti-thrombomodulin/polylysine/DNA complexes have both been used for effective gene delivery into epithelial airways (Trubetskoy *et al.*, 1992; Baatz *et al.*, 1994).

These approaches, though successful in obtaining expression of therapeutic genes in animal models, present several limitations associated with the use of polypeptides such as pLL which is known to be toxic and to have variable quality. Additional problems include aggregation in biological fluids and low levels of DNA able to formulated, pLL-DNA complexes are only practical up to about 20μg/ml which makes animal work virtually impossible (Wagner *et al.*, 1998). Modifying the poly-L-

lysine does reduce aggregation e.g. soluble DNA/transferrin-polylysine complexes can be generated at DNA concentrations upto 300μg/ml.

The limiting step in receptor-mediated gene transfer *in vitro* is the rapid degradation of DNA within the endosome after endocytosis (Curiel *et al.*, 1991). Several methods have been described for enhancing the release of DNA from the endosome before fusion with the lysosome acidifies the endosomal compartment and introduces nuclease capable of rapidly digesting the DNA. One approach has been to add non-infectious adenoviral particles (Curiel *et al.*, 1991; Curiel *et al.*, 1992; Cotton *et al.*, 1992; Wagner *et al.*, 1992a) to the transfection mixture which has led to a significant increase in transfection efficiency *in vitro*. Adenoviral particles like many other viral particles, induce endosomal lysis during the process of adenoviral infection,. Endosomal lysis by adenoviral particles is mediated by the penton protein on the surface of the virus that undergoes a change in tertiary structure upon acidification of the endosome (Seth, 1994). This change in conformation creates a structure that is capable of penetrating and disrupting the endosomal membrane and causing release of the endosomal contents into the body of the cell. Endosomal release can also be mediated by fusogenic peptides such as the influenza virus hemagglutinin peptide. The active influenza hemagglutinin peptide has a globular structure at neutral pH and assumes an amphipathic helical structure at acid pH that is capable of penetrating and disrupting endosomal membranes (Wagner *et al.*, 1992b).

Despite considerable success with receptor-mediated gene transfer using protein and endosomal release agents *in vitro*, these methods have generally been ineffective *in vivo*. The major factors limiting the *in vivo* effectiveness of polypeptide/DNA complexes may be their poor bioavailability to many target cells and their colloidal instability in physiological fluids. Bioavailability may be limited by the size of the

complex (particularly complexes incorporating adenoviral particles) as well as by aggregation and/or dissociation of these complexes in physiological fluids. Moreover, it has been difficult to achieve reproducible effects due to the intrinsic variability in the quality and size of polylysine, the covalent protein/polylysine complexes and methods used for producing formulations.

In vivo studies have revealed several different pathways for intracellular trafficking of DNA following receptor-mediated endocytosis. When plasmid DNA is delivered to hepatocytes in normal animals, DNA is cleared from the liver within several hours leading to gene expression only for several days (Wu and Wu, 1988; Wu *et al.*, 1991; Wilson *et al*, 1992). In contrast, when the same material is injected into animals after a partial hepatectomy or treatment of animals with colchicine, DNA persists in the liver for several months leading to a prolonged period of gene expression. Studies by Chowdhury *et al.*, demonstrate that partial hepatectomy leads to compartmentalisation of intact plasmid DNA within intracellular, membrane encapsulated vesicles. It was hypothesised that this persistence could be related to disruption of microtubules and the progression of the endosome along these microtubules to the lysosome. This hypothesis was confirmed with *in vivo* studies demonstrating that the administration with asialoorosomucoid/polylysine/DNA complexes leads to persistence of DNA in the liver and gene expression for 8-10 weeks (Chowdhury *et al.*, 1996).

1.2.3.3.3. Condensing Cationic Polymers

Cationic polymers associate with negatively charged DNA similarly to cationic lipids by electrostatic interactions. Several different classes of cationic polymers have

been described to enhance the uptake of DNA into cells and its release from the endosome they can be divide into two groups dendrimers and polyethylenimine.

1.2.3.3.3.1. Dendrimers

The first class are non-linear, such as polyamidoamine cascade dendrimers and Starburst™ have been synthesised. They are highly polycationic compounds that are able to condense DNA through electrostatic interactions of their terminal primary amines with the DNA phosphate groups. These complexes exhibit efficient gene delivery into a variety of cell types *in vitro* (Bielinka *et al.*, 1996). These compounds can be intact or fractured. Perfect polyamidoamine dendrimers are rigid molecules that are able to condense DNA, have high charge density, and may be further protonated in the endosome. However it appears that the highest activity is observed when the cascade polymer is imperfect (partially degraded) allowing swelling, which may explain the enhanced activity of impure dendrimers (Tang *et al.*, 1996).

1.2.3.3.3.2. Polyethylenimine

Polyethylenimine (PEI) (Boussif *et al.*, 1995; Abdallah *et al.*, 1996) is one of the most promising cationic polymers under investigation. PEIs can be linear or branched and are able to condense DNA at neutral pH. However, its branched structure, contains a proportion of tertiary amines that are protonated at lower pH. Thus, PEI becomes protonated in the endosome, which may disrupt the endosome either directly due to the membrane activity of the polycation, or possible by mechanical swelling or osmotic effects (Haensler and Szoka, 1993). PEI has been used for *in vivo* gene transfer via

different routes of administration such as lung instillation, intracranial injection and intravenous administration. PEI has been shown to transfect a large variety of cell lines and primary cultures with efficiencies at least as high as those of cationic liposomes (Bousiff *et al.*, 1996). Targeted gene delivery has also been reported by conjugating a ligand to PEI *in vitro* (Zanta *et al.*, 1997). Encouraging *in vivo* results were obtained after intracranial injection in mice (Schwartz *et al.*, 1996). The *in vivo* transfection efficiency was similar to their transfection efficiency *in vitro* for the same amount of DNA applied to neuronal cells. The highest transfection efficiencies *in vivo* was observed with PEI polyplexes bearing net charges around neutrality. Ferrari *et al.*, 1997, showed that instillation of linear PEI polyplexes into the lungs of rabbits transferred the luciferase gene more efficiently than trasfectam based gene complexes.

Gene transfer was also shown to be 10-1000 times more efficient after subcutaneous administration into tumours in mice with charge neutralised transferrin-PEI based polyplexes in comparison to naked DNA (reviewed by De Smedt *et al*, 2000). Transferrin-PEI polyplexes were used to transfect cells in culture. The possibility of reaching high transfection efficiency by using complexes with a charge ratio close to neutral is a major advantage of PEI. Electrostatic interactions between charge-neutralised polyplexes and cell surfaces are absent, a way of establishing interactions is the use of ligands e.g. transferin, galactose. PEI covalently linked to galactose (via amine formation with lactose) groups resulted in an increase in *in vitro* transfection of hepatocytes (reviewed by De Smedt *et al.*, 2000).

1.3. MICROPARTICLES FOR GENE DELIVERY

While viral vectors and liposomes have been used in a majority of the gene therapy trials so far, results fail to show significant improvement compared to conventional methods of treating genetic disorders. Thus, novel vehicles for gene transfer are being developed and assessed in appropriate animal disease models. Microparticles are one class of vehicles currently being examined for gene delivery.

Microparticulate systems can be applied to a wide diversity of routes for administration e.g. pulmonary, intratumoral, oral and nasal to mention a few. Their main advantage is that they can be used to provide non-invasive controlled drug delivery for a period of time and do not need to be removed once the therapy is complete. However, several factors need to be considered when formulating microparticles for each of these routes of administration, these are listed in Table 1.1, as each of these routes of administration will require properties specific to the individual route.

Table 1.1 Factors to be considered prior to introduction of microparticles for use *in vivo* (Dass and Burton, 1999).

- Structure and size of particles
- Surface properties of particles, such as charge and texture
- Degradability of the matrix during storage and persistence *in vivo*
- Toxicity of the particles when intact and when degraded
- Antigenicity/immunogenicity of the particles
- Physiochemical features of the drug to be loaded, such as hydrophilicity
- Stability of the drug during incorporation, storage and use
- A rational dose regimen determined from animal and clinical studies
- Mechanism of release of the drug from the carrier
- Administration via the carrier is at least as therapeutically efficient as free drug

Microparticles are generally fine spherical particles with diameters less than 125 μm to be of any therapeutic value, and can be divided into two categories: (a) microspheres, homogenous or monolithic in which the drug is dissolved or dispersed throughout the polymer matrix, solid dispersion and (b) reservoir type microcapsules in which the drug is surrounded by the polymer matrix in the mononuclear or polynuclear state, finally, (c) in some systems the drug is adsorbed or chemically conjugated on the surface of the polymer or entrapped inside the pores or channels in the matrix (Okada and Toguchi, 1995).

Table 1.2. Factors affecting drug loading of microspheres (Dass and Burton, 1999).

- Chemical composition of sphere matrix
- Size of spheres
- Degree of porosity of matrix
- Micropshere formulation procedure
- Type of bonding between drug and microsphere surface
- Influence of other agents in the drug loading mixture
- Concentration of drug in the loading mixture
- Temperature at which drug is loaded

Due to the versatility of the matrices available drugs of varying characteristics can be loaded. The incorporation of drugs and release of drugs from these microparticles are dependent on various factors, which are listed in table 1.2 and 1.3 respectively.

Table 1.3. Factors affecting drug release from microspheres (Dass and Burton, 1999).

• Chemical composition of sphere matrix
• Temperature at which spheres are formulated
• Treatment of postmanufactured preloaded spheres with heat
• Chemical treatment of postmanufactured preloaded spheres
• Percentage of drug loaded
• Type of bonding between drug and sphere surface
• Chemical treatment of loaded microspheres
• Temperature of eluting buffer
• Type of *in vitro* release system

These factors can be taken into account when formulating a gene delivery incorporating microparticles with plasmid DNA.

1.3.1. Matrix Composition of Microspheres

The choice of a matrix depends on several factors, including the chemical nature of the drug to be loaded, release characteristics from matrix, drug-loading capacity of spheres, and ease and cost of preparation. A variety of materials are used for fabrication of biodegradable microparticles, including proteins, polysaccharides, and synthetic polymers.

1.3.1.1. Proteins

The major advantages in using protein-based microspheres is their compatibility and degradability. Biodegradable microspheres release carried drug in three phases (a) initial burst effect due to release of weakly attached drug on surface of spheres, (b) second phase, where drug release is slow due to medium diffusing slowly into the polymer matrix, whereby degradation occurs and drug diffuses out into the medium, and (c) the final release phase, when matrix becomes water soluble, leading to an erosion of the residual matrix and eventual collapse of the spheres (Singh *et al.*, 1996). Functional groups such as amine (-NH2) and carboxylic (-COOH) make proteins suitable for microspherical drug delivery. Albumin is prevalently used, although fibrinogen (Miyazaki *et al.*, 1986), gelatin (Narayani and Rao, 1996a), haemoglobin (Chen *et al.*,1988), transferrin (Chen *et al.*, 1988), fibrin (Ho *et al.*, and casein (Chen *et al.*, 1994) have also been used for microsphere formulation.

Albumin microspheres have been used for delivering epirubicin into mice (Novotny and Zinek, 1994), and adriamycin into rats (Goldberg *et al.*, 1992) with greater tumour suppression compared to free drug. Albumin microspheres have also been investigated as a inhaled drug delivery system (Zeng *et al.*,1995).

1.3.1.2. Polysaccharides

Polysaccharides such as alginate, dextran and starch have been used for drug delivery. Degradable starch microspheres (DSMs) have also been used towards tumour therapy. If the blood supply to a tumour can be located and is accessible by catheter, regional perfusion with drugs can be affected. To enhance regional drug delivery DSMs

of 15 to 80 μm in size are infused into the arterial blood supply of a tumour (Dass *et al.*, 1998).

1.3.1.3. Synthetic Polymers

Synthetic polymers are generally superior with respect to the reproducibility of the product compared to natural polymers. Synthetic polymers are highly lipophilic and dissolve in organic solvents in which lipophilic drugs can be dissolved, and hydrophilic drugs can be suspended or emulsified as an aqueous solution to prepare the microparticles. A major advantage of some of these polymers is that their degradation rate can be controlled through manipulating either the molecular weight of a homopolymer, or hydrophilicity and composition of a copolymer.

Microspheres with polystyrene-based matrices usually load and release drugs via ion-exchange resins depending on the availability of competing ions and diffusion rates of ions and drug molecules into and out of the resin matrix (reviewed by Dass and Burton, 1999). Factors influencing drug release include the size and concentration of the competing ions, selectively for the resin of the different ions involved, and the porosity or degree of cross-linkage of the resin matrix. One advantage of ion-exchange microspheres is that the drug is loaded postmanufacture and is not exposed to the usually harsh conditions of sphere formulation. This is especially important when fragile agents such as long strands of DNA are loaded. However, only drugs with an overall charge may be loaded on these microparticles.

1.3.2. Generation of Microparticles

The most basic methods for formulating microparticles are the solvent evaporation and solvent extraction (coacervation) methods. The solvent evaporation method involves the following steps, firstly, the polymer e.g. PLGA or PLA is dissolved in an organic solvent e.g. methylene chloride or ethyl acetate. The aqueous or solid drug is then added, and the solution is mixed by sonication or homogenisation to form the primary emulsion (solid or water-in-oil). This emulsion is then transferred to water containing an emulsifying agent e.g. poly(vinyl alcohol). Mixing of the primary emulsion in the water phase produces the microspheres, resulting in a secondary emulsion (solid or water in-oil-in-water. The final emulsion is diluted with excess water to facilitate removal of the organic solvent in the oil phase, and the microspheres are then dried (Cleland, 1997).

Where as the solvent extraction method involves similar steps in the initial stages with dissolving the polymer in an organic solvent. The aqueous or solid is then added, and the solution is mixed by sonication or homogenisation to form the primary emulsion as before. This emulsion is then transferred to a nonsolvent, i.e. a solvent in which the polymer has a low or negligible solubility e.g. silicone oil. Mixing of the primary emulsion in the nonsolvent produces the microspheres, resulting in a secondary emulsion (solid or water in-oil-in-oil). Another nonsolvent e.g. heptane is added to the final emulsion to extract the organic solvent from the first oil phase. The excess solvents in the supernatant are then removed, and the final microspheres are dried (Cleland, 1997).

Recently, several new approaches have been developed to produce an improved process for encapsulation of proteins in microparticles. Many of these approaches have

focused on methods that do not denature the protein e.g. operation of the microencapsulation process at low temperatures and in the absence of a water phase should result in increased stability of the protein during the process. One method employed the spray freeze drying of proteins to obtain solid protein particles of 2-5 μm in diameter. The solid protein was then suspended in the polymer phase, and the suspension was sprayed into a container with solid ethanol and liquid nitrogen. The microspheres formed during the spraying process settled onto the solid ethanol, and the solution was then warmed to -80°C. The microspheres were suspended in the cold, now liquid, ethanol to extract the organic solvent from the polymer phase. After repeated washing with cold ethanol, the microspheres were dried by lyphilization. This process was claimed to provide a high encapsulation efficiency and enhanced stability of the protein (reviewed by Cleland, 1997).

The conventional spray drying approach at high temperatures resulted in microspheres that tended to agglomerate and have variable morphology depending upon the polymer. The high-temperature spray drying process may cause significant protein denaturation (Pavanetto *et al*, 1993).

Another method that utilised low-temperature processing without an aqueous phase involved supercritical fluid extraction techniques. The polymer, which was dissolved in an organic solvent, was sprayed into a continuous phase of supercritical carbon dioxide. The carbon dioxide acts as a nonsolvent for the polymer phase and extracts the organic solvent. The microsphere size should correlate to the density of the carbon dioxide phase (e.g. more dense, larger microspheres). This process should also provide high encapsulation efficiency and improved protein stability (Randolph *et al*, 1994).

Other new processes have involved modifications of the solvent evaporation method. To produce a more homogeneous emulsion and stabilise the protein, surfactants have been added to the protein phase. It has been suggested that the addition of hydrophobic ion pairs to the protein phase allows a more homogeneous mixture of the protein in the polymer phase and provides stabilisation of the protein (review by Cleland, 1997). Multiphase systems may also protect the protein from denature. One type of multiphase system involves the suspension of aqueous drug in a heavy oil phase (primary emulsion), which is then emulsified in a light oil phase containing the polymer (secondary emulsion), and the secondary emulsion is then added to an aqueous solution to extract the light oil e.g. methylene chloride and form a triple emulsion (Iwata and McGinity, 1993).

Surfactants have also been added to the polymer phase to alter the release properties of the microspheres. The addition of non-ionic surfactants such as pluronics (block copolymers of ethylene oxide and propylene oxide) provides a reduced initial burst of protein release from these microparticles is further reduced by the addition of Polyethylenimine, which coats the microspheres and interacts with the protein to inhibit release (Park *et al*, 1992b). However, these methods in their present form do not provide high encapsulation efficiencies, and hydrophobic proteins may remain entrapped in the surfactant-polymer microspheres.

1.3.3. Microparticles and DNA Delivery

There are various studies evaluating the potential of microparticles for DNA transport. For instance, single and double stranded DNA were encapsulated in gelatin microspheres (Cortesi *et al.*, 1994). These researchers immobilised fragments of 144

base-pairs length into microspheres and characterised the *in vitro* release. Encapsulation efficiency for both types of DNA was at least 85% under varying loading conditions. Release was byphasic for both types of DNA, with an initial rapid release followed by a slower release phase.

Ion-exchange microspheres have been used to bind and release plasmids without degradation of the nucleic acids (Dass *et al.*, 1996, 1997b). Polyanhydride microparticles were used to deliver plasmid DNA encoding ß-galactosidase into rats via the oral cavity (Mathiowitz *et al.*, 1997). Encapsulation of the plasmids within the microparticles allowed greater expression of the gene in both the small intestine and the liver. Increased expression may be attributed to delayed release of DNA due to slow dissolution of sphere matrix, increased DNA uptake into cells due to physical contact of microspheres with target cells, and resistance proffered by complexation of DNA with the microsphere matrix. However, transfection was not reproducible in the small number of animals studied, and more importantly, polyanhydride did not cause an immunological reaction with mammalian tissue, as documented elsewhere (Edwards *et al* ., 1997).

In an attempt to decrease the dose of adenoviral vectors for gene transfer into glioblastomas, recombinant viral units were encapsulated in poly(lactic-glycolic) microspheres (Beer *et al.*, 1997). Gene transfer was detected up to 15 days in HeLa cells, as was a dose-dependent increase in cytotoxicity of 9L gliosarcoma cells. A dose-dependent increase in gene expression in mice was noted at 7 days postinjection of microspheres.

Gold particles of diameters between 1-3 μm have been used for bombardment of genes into tumours in mice (Sun *et al.*, 1995). DNA was coated onto particles with a capacity of 2.5 μm per milligram particles. Mice were placed with the tumour

surgically exposed and directly in the path of electronically accelerated particles. Expression of foreign genes was observed in the tumour and not in other tissues analysed.

1.3.3.1. Microparticles for Lung Delivery

There is a wealth of knowledge on delivery of macromolecules to the airways by inhalation (Patton, 1996). The requirements for deposition of particles in the airways have been known for many years, and have been used to design inhalation systems for low-molecular weight drugs. Generally particles of 2-5μm are retained in the airways, so it will be necessary to disperse the gene delivery system within particles of these dimensions. Formulations of lipoplexes have been developed for aerosol formation by nebulisation (Schwarz *et al.*, 1996; Eastman *et al.*, 1997). It is necessary to select an appropriate nebulizer, which does not create too much force of shear, and also screen formulations for stability under conditions of shear. Higher concentrations of DNA in the formulation were made possible, in the studies of Eastman *et al.* (1997), by incorporation of some PEGylated lipids. The critical issue in delivery to the lung will be the interaction of the formulation with the biological barriers presented by the lung epithelium. Though it has been possible using gene therapy to treat cystic fibrosis in mouse models of the disease (Alton *et al.*, 1993; Dorin *et al*, 1996), the results of the trials in patients have been less encouraging.

Gene expression systems have been introduced to the lungs of animals either by inhalation or by aerosolization (Brigham *et al.*, 1989, 1993; Hazinski *et al.*, 1991; Yoshimura *et al.*, 1992; Logan *et al.*, 1995) (Stribling *et al.*, 1992; Alton *et al.*, 1993; Canonico *et al.*, 1994; MacLachlan *et al.*, 1994), and more recently attempts have been

made to optimise the formulation of lipoplexes for gene delivery to airways epithelia (Fasbender *et al.*, 1995; Lee *et al.*, 1996).

Another, recent method for aerosol gene delivery is a system designed by Schuster *et al.*, 1997. The AERx™ was designed as an alternative to nebulization, to avoid shear degradation via nebulization. Sorgi *et al.*, 1998, have formulated DNA into an artificial viral envelope (AVE™) vehicle in which plasmid DNA is condensed and protected with a bilayer of anionic liposomes. This AVE/DNA vehicle is then delivered via the AERx™ aerosol delivery system, which generates a respirable aerosol cloud by a single extrusion through an array of precise micron-sized holes.

However, considerable research and development has yet to be carried out regarding delivery of therapeutic genes to the lung via the use of microparticle formulations. Biodegradable microspheres of the appropriate size distribution have been shown to lodge within the capillary networks of the lung and release their bio-active agent into neighbouring tissue by a process of diffusion and biodegradation (Willmott *et al.*, 1984). A similar approach was used by Schaefer and Singh, 1997, who produced poly-lactic acid microspheres loaded with etoposide a chemotherapeutic agent for intravenous administration by a simple process of mechanical entrapment in the capillary beds of the lung for passive targeting of lung tumours. Specific targeting of microspheres to the lung for tumour thereby will greatly increase the concentration of the etoposide locally and decrease the concentration systemically, thereby eliminating or lessening the potent side effects.

1.3.3.2. Particulate DNA Vaccines

Direct DNA inoculation, without the use of any viral vectors, is among the most interesting new additions to vaccine development. This approach is conceptually simple and can be viewed as the most elegant form of sustained protein delivery. Plasmid DNA encoding the antigenic protein is directly introduced to the tissue either by intramuscular or intradermal injection or by bombardment of DNA-coated gold particles through a high-voltage gene gun. After taking up the plasmid by non-specific endocytosis or cell membrane disruption, the host cells produce, process, and present the encoded antigen to the antigen presenting cells (APCs) in a manner similar to that which would happen during the course of natural infection.

Direct DNA vaccination has generated both humoral (Mor *et al.*, 1995) and cellular (Pertmer *et al.*, 1995; Ulmer *et al.*, 1994) protective immunity to a number of pathogens. Direct inoculation of a tumour-associated antigen gene has also been used to enhance the immunogenicity of tumour cells and stimulate long-term systemic immunity (Conry *et al.*, 1995). For example delivery of the IL-6 gene by a gene gun into the tumour site reduced methylcholanthrene-induced fibrosarcoma growth, and similar inoculation of the TNF-α and IFN-γgenes have prolonged the survival of Renca tumour-bearing mice (reviewed by Zhao and Leong, 1996). Relatively high efficiency of vaccination is one of the attractive features of DNA vaccines, although an effective delivery of the DNA is crucial.

As DNA is not an effective delivery alone for reasons covered through this chapter, delivery systems that protect DNA and target it to antigen-presenting cells are essential for the successful design of plasmid DNA vaccines. Particulate carrier systems such as microspheres offer promise of effective delivery of plasmid DNA for

vaccination. The potential advantages of microparticles for vaccine development are shown in table 1.4. As well as offering the possibility of offering a controlled rate of release of the entrapped antigens they enhance the immunogenicity of the DNA vaccine.

Table 1.4. The potential advantages of microparticles for vaccine development.

• Safety: biodegradable and biocompatible polymers
• Acceptable for administration to man
• Controlled-release might enable the development of single-dose vaccines
• Adjuvants might be entrapped simultaneously in the microparticles
• Many antigens can be administered by mucosal routes, including oral delivery
• Antigens are protected from degradation in the intestine
• Antigens are targeted to lymphoid tissue
• Microparticles induce serum and secretary antibodies
• Microparticles induce cell-mediated immunity
• Freeze-dried formulations, with enhanced stability for entrapped antigens
• Large scale manufacture of microparticles has already been achieved

The adjuvant effect achieved through the association of antigens with polymeric microparticles has been repeatedly demonstrated (O'Hagan, 1997). Encapsulation of antigens into microparticles, including submicron particles, promotes their entry into lymph nodes and provides a high local concentration of antigen over an extended time-period. Microparticles also promote the interaction of encapsulated antigens with APCs e.g. macrophages. The poly(lactide-co-glycolides), biodegradable and biocompatible polyesters, are primary candidates for the development of microparticles as vaccines, because they have been used in man for many years as controlled delivery systems for peptide drugs. However, the adjuvant effect achieved by the encapsulation of antigens into poly(lactide-co-glycolide) microparticles has been demonstrated only relatively recently (O'Hagan *et al* 1991a). Particle size was shown to be an important factor affecting immunogenicity, because smaller microparticles (<10 μm) were significantly

more immunogenic than larger particles (O'Hagan, 1993). The adjuvant effect of microparticles can also be enhanced by co-administration with additional adjuvants (O'Hagan, 1991b). Recent studies have shown that microparticles exert an adjuvant effect for cell-mediated immunity, including the induction of cytotoxic T-cell responses, (which are desirable for the immunotherapy of cancer) after both systemic and mucosal administration (Maloy *et al.*, 1994; Moore *et al.*, 1995). The induction of cytotoxic T-cell responses are important for the eradication of virally infected cells and for immune responses against alternative intracellular pathogens.

1.4. AIMS AND OBJECTIVES

Microparticulate systems can be applied to a wide diversity of routes for administration e.g. pulmonary, intratumoral, oral and nasal to mention a few. Their main advantage is that they can be used to provide non-invasive controlled drug delivery for a period of time and do not need to be removed once the therapy is complete.

The objective of this research was to investigate the potential of spray dried microparticles produced by an established spray drying procedure at Quadrant Healthcare (previously, Andaris Ltd) to act as carriers for therapeutic DNA for pulmonary and intratumoral delivery. In **Chapter 2** the possibility of producing reproducible soluble mannitol microparticles as carriers for DNA/lipid complexes for lung delivery through utilising Quadrant's proprietary spray drying method was investigated.

Transfection *in vitro* with reporter genes is a good assay to assess biological activity of plasmid DNA and efficiency of gene delivery vectors whether they are lipids, polymers or microparticles. However, when trying to detect very low levels of transfection, a very sensitive assay is needed. ß-gal is a very popular reporter gene, however as mammalian cells have apparent galactosidase activity, the levels of basal activity are quite high. This results in interference when trying to detect low levels of transfection activity. Hence an alternative assay was investigated in **Chapter 3**, based on the luciferase reporter plasmid. In **Chapter 4** the potential of spray-dried cross-linked albumin microparticles (produced by Quadrant's proprietary spray drying method) to be used as carriers for DNA vaccines was investigated.

In the final results chapter, **Chapter 5** the location and stability of the cross-linked albumin microparticles in tumour tissue was investigated *in vivo* after intratumoral injection, as was the relationship between the presence of the albumin microparticles *in vivo* and the number of macrophages present in tumour and muscle tissue.

CHAPTER 2

EVALUATION OF MANNITOL MICROPARTICLES AS GENE THERAPY VECTORS FOR THE LUNG

2.1. INTRODUCTION

The pulmonary route of administration, is attractive for the delivery of pharmaceuticals due to the large surface area, high degree of vascularisation and ease of accessibility, which the lung provides. The same holds true for the delivery of proteins, peptides and genes. Several DNA-based delivery systems, i.e. the adenovirus (Rosenfeld *et al*, 1994), adeno-associated virus (Flotte *et al*, 1993) and liposomes (Schreier and Sawyer, 1996) have been used, to varying degrees of success, to deliver genetic material to the lung. However, most have been administered by direct instillation (Brigham *et al*, 1989; Logan *et al*, 1995) or aerosolisation (Stribling *et al*, 1992; Alton *et al*, 1993; McLachlan *et al*, 1995). Direct instillation of the vector into the lung is impractical and undesirable as a manner of delivery for maintenance dosing. Most aerosolised vectors are in liquid form and are aerosolised by nebulisation, which is inefficient and irreproducible and may induce shear degradation of the plasmid DNA (Schwarz *et al*, 1996; Eastman *et al*, 1997).

Another alternative, is to formulate a DNA-based delivery system as a dry powder formulation which could be administered using an existing dry powder delivery device, many of which are known to be fairly efficient and reproducible in their mode of delivery. One way of formulating the plasmid DNA into a dry powder formulation, may be to encapsulate the DNA-based vector in a protective matrix formed by spray-drying with a sugar for example, thereby enhancing DNA stability and protecting the

biological activity of the plasmid. The method of spray drying will be a reproducible method which can produce microparticles of an appropriate size, which enables them to be inhaled and deposited in the lungs.

This chapter investigates the possibility of using Quadrant's proprietary spray drying method to produce soluble mannitol microparticles carrying a DNA-lipid complex as a dry powder formulation. Firstly, it was necessary to investigate the feasibility of producing uniform reproducible microparticles via spray drying. Secondly, survival of the plasmid after undergoing the spray drying process was investigated. Finally, attention was given to the stability of the plasmid and its biological activity after under going spray drying.

2.2. METHODS

2.2.1. DNA Preparation

2.2.1.1. Bacterial strain

Escherica coli strain XL1-Blue (Stratagene), which has resistance to tetracyline was used for propagation of plasmids.

2.2.1.2. Deoxyribonucleic Acid (DNA)

Two types of plasmids, pRSV*lacZ* and pCMV*lacZ* were used. Both plasmids contain the E.coli *lacZ* gene, which encodes the ß galactosidase enzyme.

2.2.1.3. pRSVlacZ

pRSV*lacZ* was obtained from Dr Ogilvie (Zeneca Pharmaceuticals). It is a 7.8 kb plasmid where the E.coli *lacZ* gene is under the transcriptional control of the Rous sarcoma virus (RSV) long terminal repeat promoter/enhancer sequence. The RSV promoter is a moderate to high strength promoter causing moderate levels of transgene expression in mammalian cells. The plasmid map for pRSV*lacZ* may be found in appendix A.

2.2.1.4. pCMVlacZ

pCMV*lacZ*, a 7.2 kb plasmid was obtained from Clontech, UK. In this vector the *E.coli lacZ* gene is under the transcriptional control of the human cytomegalovirus (CMV) immediate early promoter/enhancer element. The CMV promoter is a strong

viral promoter causing high levels of transgene expression in mammalian cells. The plasmid map for pCMV*lacZ* may be found in appendix A.

2.2.1.5 Plasmid Propagation, Isolation and Purification

A single colony of *E.coli* XL1-Blue isolated from an LB agar plate supplemented with ampicillin and tetracycline (50µg/ml and 12.5µg/ml respectively) was incubated into 10ml of LB broth (1° culture) containing the same antibiotics. The 1° culture was grown for 8 hours at 37°C in a shaking incubator at 300rpm (New Brunswick Scientific), then a 5ml aliquot from the 1° culture was expanded in 500mls of LB broth overnight. The plasmid was isolated according to the alkaline lysis method and the cell lysate purified by anion exchange chromatography using the Plasmid Mega Kit (Qiagen Inc, UK) following the manufacturer's protocol. The isolated DNA was dissolved in sterilised Milli-Q water and stored at -20°C.

2.2.1.6 Sample Purity and Quantification

For quantification of plasmid DNA, a 10µl aliquot of sample was diluted to 1ml with sterilised Milli-Q water and the absorbance of the solution measured at 260nm (A_{260}) and 280nm (A_{280}). The concentration of DNA in the original solution was calculated as follows:

Concentration of DNA (µg/ml) = Dilution factor × 50[1] × A260

[1] based on the assumption that a 50 µg/ml solution of double standard DNA has an absorbance of 1 at 260nm (Sambrook *et al*, 1989; reviewed by Felgner, 1997).

The purity of DNA was calculated as follows:

$$\text{Ratio} = A_{260} / A_{280}$$

Ratios between 1.8 and 2.0 (which indicates high purity) were obtained consistently.

2.2.1.7. Sample Identification

A sample of purified DNA was linearised with a restriction endonuclease (New England Biolabs, UK). *Xba* I for pRSV*lacZ* or *Eco*R I, *Pst* I, *Not* I for pCMV*lacZ* enzymes for one hour at 37 °C and then loaded onto a 1% agarose gel (Ultrapure grade, Gibco, UK) in 1x TBE buffer. The gel was subjected to electrophoresis in the same buffer at 80 volts for one hour and 45 minutes against marker DNA (*Eco*R I/ Hind III cut DNA, see Appendix) of variable length to confirm the size of isolated DNA. Control of indigested DNA were also run to exclude the presence of contamination genomic DNA or RNA in samples (*appendix B7*).

2.2.2. Gene Delivery Vectors

2.2.2.1. DOTAP

N-[-1-(2,3-Dioleolyoxy)propyl]-N,N,N-trimethylammonium-methylsulphate (Avanti Polar Lipids, USA). DOTAP a cationic lipid presented in this case as a powder, was used to prepare liposomes as described in section 2.2.2.3.

2.2.2.2. DOPE

Dioleoylphosphatidylethanolamine (Avanti Polar Lipids, USA). DOPE a neutral lipid presented in this case as a powder, was used to prepare liposomes as described in section 2.2.2.3.

2.2.2.3. Preparation of Liposomes

2.2.2.3(a) DOTAP Liposomes

Liposomes were prepared by the reverse phase evaporation method (Szoka & Papahadjopoulos, 1978, Hug & Sleight, 1991). DOTAP powder was dissolved in chloroform to produce a 5 mg/ml stock solution. An appropriate volume of solution was transferred to a clean 4 ml HPLC glass vial. The chloroform was evaporated, by bubbling nitrogen gas, leaving a thin lipid film on the glass wall. Residual amounts of chloroform were removed by placing vials under high vacuum overnight. The lipid film was subsequently re-dispersed in sterile 95% w/v mannitol solution to give a 5mg/ml solution of large multilamellar vesicles by vortexing following preparation. Liposomes were stored at 4 °C and used within 7 days.

2.2.2.3(b) DOTAP:DOPE Liposome

Liposomes containing a 50:50 weight mixture of the cationic amphiphile DOTAP to the neutral lipid DOPE, were prepared by the reverse phase evaporation methods described in section 2.2.2.3(a). The lipid film was re-dispersed in sterile 95% w/v mannitol solution to give a 5mg/ml solution of large multilamellar vesicles by

vortexing. Following preparation, liposomes were stored at 4 ° C and used within 7 days.

2.2.3. Spray Drying

Spray drying is a one-step process that converts a liquid feed to a dried particulate form. In a typical application, the fluid is first atomised to a spray form, which is in thermal contact with a hot gaseous medium. The large surface area of contact results in rapid evaporation of the droplets to form dried solid particles/granules, which are then separated from the gas by means of a cyclone, electrostatic precipitator, or bag filter (Sacchetti and Van Oort, 1996).

The spray drying process can be broken down into four fundamental stages: circulation of drying gas, atomisation, drying and separation. The first consideration is whether it is an open or closed cycle operation. An open cycle system is a system where the drying gas, usually air is vented out into the atmosphere; where as in a closed cycle system the drying gas (usually nitrogen with an oxygen content maintained less than 5%, below level required for ignition) is recirculated.

Atomisation is a process whereby a liquid is broken up into a collection of droplets. Since the surface area and surface free energy are increased upon spray formation, atomising a fluid requires work. The amount of work and the manner in which it is done (i.e. nozzle design) on the liquid can affect the droplet sizes, and can thus provide flexibility and control in generating a desired particle size distribution. There are many methods of atomising a liquid, the chief ones of which can be classified according to the way energy is transferred to the liquid: centrifugal (rotary), pneumatic (air-assist), high pressure, effervescent, ultrasonic and electrostatic. For more details of how the afore mentioned atomisation methods work see Sacchetti and Van Oort (1996).

Once the liquid is atomised it is in intimate contact with the heated gas, and evaporation of the drops occurs, accomplishing drying. During their residence in the main chamber these droplets dry to form solid particles/granules. This drying step is critical for several reasons. Although initial droplet size is determined by atomisation variables, it is during the drying process that solid particles form. The final size, shape, density, crystallinity and moisture content of the particles is affected by the conditions in the drying chamber (Sacchetti and Van Oort, 1996).

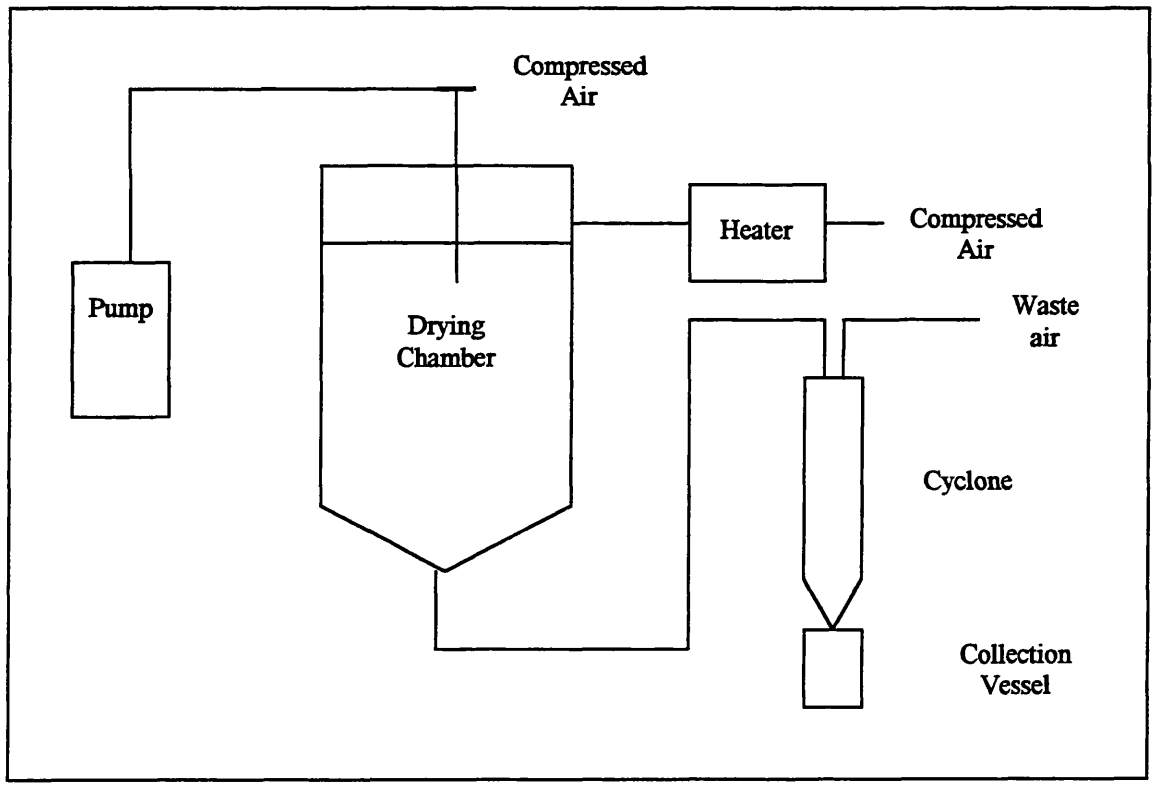
Finally, after the spray-dried powder is formed, it must be separated from the circulating gas medium. The three methods for separation are cyclone separators, bag filters and electrostatic precipitators. In a conventional cyclone the tangential entry produces a downward spiralling vortex with a central core of upward flowing gas. The circulation of the velocity field produces a centrifugal force on the entrained spray-dried particles. Thus, the particles migrate radially outward, will impact on the cyclone wall and will be removed from the gas flow with a certain collection efficiency. The downward velocity component of the spiralling motion results in powder collection in the vessel attached to the bottom of the cyclone. Bag filters, as the name implies is a woven fabric used to remove spray-dried particles by filtration from the gas flow. The fabric is attached to the output of the drying chamber as a stand-alone unit or is used after a cyclone in a two-stage recovery to collect the ultrafine particles. Electrostatic precipitators used in spray drying are normally of parallel plate design with a discharge electrode to ionise the gas stream. The spray-dried particles acquire charge and migrate to the grounded plates for removal from the gas flow (Sacchetti and Van Oort, 1996).

This variety of atomisation, drying and separation techniques enables spray drying to be adapted to many applications.

2.2.3.1. Spray Drying Method

The spray drying process used at Quadrant Healthcare plc (formerly Andaris Ltd) Nottingham, UK, was a low temperature 'open cycle' spray drying process, with pneumatic atomisation (twin-fluid), in this case achieved with an external mixing nozzle. Fig 2.1 shows a schematic layout of the "open cycle" spray drying process and the components special to this particular set up. The lipoplex formulations were spray dried aseptically, using the afore mentioned process in conjunction with a Research Scale Spray Dryer, proprietary instrument of Andaris. This instrument is designed as part of a scalable system, which offers better simulation of a process-scale than laboratory bench spray dryers. The conditions of the process were monitored and documented (see results).

Figure 2.1 Schematic layout of the Research Scale Spray Dryer and the 'Open Cycle' process used at Quadrant Healthcare plc.



Briefly, the air was exhausted directly into the ductwork of the sterile chamber. The drying gas was heated to the desired temperature, which was monitored and maintained by a thermocouple and feedback system. The drying gas passed through the distributor plate and passed into the main chamber, where it was mixed with the spray generated by the atomiser. The enormous surface area of the spray and its intimate contact with the heated gas result in rapid drying and solidification of the feed substance. The majority of the mass passed through the cyclone, where it was separated from the gas flow and collected in the vessel. The filter bag removed the ultra fine particles before exhausting the moist gas to the atmosphere. Exact conditions are detailed later in this chapter.

The product was recovered from the spray drier by carefully brushing through all the chambers and collating all the powder in the collection vessel. It was then transferred to a sterile vial under sterile conditions.

2.2.3.2. Preparation of Formulae for Spray Drying

The minimum volume that could be spray dried in the mini-spray dryer was approximately 2 ml of a solution with a minimum solid content of 500mg, to ensure recovery of at least 10% of the product. These practical constraints were particularly important for DNA formulation. DNA was difficult to produce, time consuming, very expensive and only small amounts (2.5 mg per lot) could be produced at bench scale. Therefore the formulations used in this study were adapted with the minimum volume in mind. Since a maximum of 2.5mg of plasmid DNA could be produced per batch it was appropriate to use DNA at 0.5 mg/ml for spray drying. Control batches were spray dried for each formulation consisting of cationic lipid in mannitol solution. Spray drying experiments were carried out in duplicate.

Tables 2.1a-b show the formulations used for spray drying. Two batches were prepared in each case.

(a)

	Control Formulation 1 (mg/4ml)	Formulation 1 (mg/4ml)
pRSV*lacZ*	0	2
DOTAP	10	10
Mannitol	390	388

(b)

	Control Formulation 2 (mg/4ml)	Formulation 2 (mg/4ml)
pRSV*lacZ*	0	2
DOTAP:DOPE(w/w)	10	10
Mannitol	390	388

2.2.4. Physical Characterisation Methods

2.2.4.1. Light Microscopy

A small number of particles were taken and placed on a microscope slide to which a few drops of acetone were added before a cover slip was placed on the slide. The slide was then visualised under a light microscope at a magnification of x400. Images were taken of the microparticles, this was carried out for each batch produced.

2.2.4.2. Particle Size Analysis

Particle sizing was achieved using the Small Volume Module Laser Sizer LS23 (Coulter). 18 mg of each sample was placed in 6 ml of acetone, then sonicated for 30 seconds to deaggregate the particles before sizing. A saturated representation of each sample was introduced into the cell containing isobutyl alcohol as the dispersant, dropwise using a pastuer pipette. The Laser Sizer uses the principle of Fraunhofer Diffraction Theory. Basically, the laser beam (i.e. a monochromatic light source) hits the sample of suspended particles. The light is diffracted at right angles and the diffracted light beams are focused onto a series of concentric rings, each one associated with a particular range of particle sizes. From here the signals are amplified by means of a photomultiplier tube and digitised by computer. Particles between 0.04-2000 μm can be measured. Hence particle size measurements are calculated by volume and represented as volume median diameter (VMD). The values at 10, 50, and 90% levels represent the percentage cumulative undersize for the samples tested.

2.2.4.3. Electrophoresis

Identification and integrity of the plasmid after spray drying was assessed, by electrophoresis. A sample of Formulation 1 prepared for transfection was loaded onto a 0.8% agarose gel along with a sample of DNA from the original stock of the same batch. The gel was run in 1x TBE buffer at 80 volts for 1 hour and 45 minutes against marker DNA (*Eco*R I/Hind III cut λDNA).

2.2.5. Cell Culture

All biochemicals were of analytical grade and purchased from Sigma Chemicals, UK except where indicated.

2.2.5.1. Solutions

2.2.5.1.1. Water

Fresh Milli-Q water was used for the preparation of all culture media and solutions and was obtained from the Milli-Q Plus PF (Millipore UK Ltd).

2.2.5.1.2. Phosphate buffered saline (PBS)

PBS tablets without magnesium or calcium ions were obtained from Oxoid Ltd, UK. One tablet was dissolved in 100ml Milli-Q water with a final pH of 7.3 and sterilised by autoclaving at 121°C for 15 minutes.

2.2.5.1.3. Ethylene diamine tetraacetic acid (EDTA)

A 0.02% w/v solution of EDTA was prepared in PBS and sterilised by autoclave.

2.2.5.1.4. Sodium bicarbonate and Sodium Hydroxide

Solutions of 7.5% w/v sodium bicarbonate and 1M sodium hydroxide were prepared individually with Milli-Q water and sterilised by autoclaving.

2.2.5.1.5. Trypan Blue

Trypan Blue used for assessing cell viability, was obtained from BDH Laboratory Reagents Ltd and was prepared as a 0.1% w/v solution in PBS. .

2.2.5.2. Culture Media and Additives

Culture media were prepared aseptically according to the formulae shown below, and stored at 4 °C and used within 3 weeks of preparation.

		Medium (ml)
MEM 10x		50
FCS		55
MEM non essential amino acids		5
L-glutamine		5
Penicillin/Streptomycin		5
7.5% w/v NaHCO3		15
Milli-Q Water	to	500
1M NaOH adjust to pH 7.4		

All solution used above were obtained from Gibco BRL, Paisley. Eagle's Minimum Essential Medium - Eagle's (MEM) with Earle's salts was obtained as 10x concentrate and contained phenol red as an indicator. MEM non-essential amino acids solution, the antibiotics L-glutamine (200mM) and penicillin (10000 IU/ml) / Streptomycin (10000 µg/ml) were obtained as 100x concentrates.

Foetal calf serum (FCS) from various suppliers was batch tested to determine which provided optimum growth of cells. Batch 06Q7554F was found to support growth of all cells and was thus used.

2.2.5.3. Equipment

2.2.5.3.1. Laboratory Equipment

All aseptic techniques were carried out in laminar flow cabinets (MDH Ltd) designed for vertical re-circulation of air. The cells were cultured in a LEEC PF2 anhydric incubator (Laboratory and Engineering Company) with a pre-set temperature of 37 °C.

Growing cell cultures were viewed daily under an inverted light microscope (WILD MS, Wild Heerbrugg Ltd). For determination of cell concentration in suspension, appropriately diluted samples were counted on a standard double grid haemocytometer (Neubauer 0.1 mm Dept, Weber, UK).

2.2.5.3.2. Disposable Items

Sterile 75 cm^2 and 175 cm^2 filter top tissue culture flasks were obtained from Falcon Becton and Dickson and Co, UK. Six-well plates were obtained from Nunc, (Denmark). Cryopreservation ampoules used for storing stocks of frozen cells in liquid nitrogen were obtained from Corning, UK.

2.2.5.4. Cell Culture Methods

2.2.5.4.1. Cell line

B16 cells, a murine melanoma cell line was donated by L.R. Kelland, Institute of Cancer Research, Sutton. This line was used for transfection studies because it was

known to express high levels of reporter genes under the control of the CMV or RSV promoter.

2.2.5.4.2. Cell line maintenance

B16 cells were maintained at 37 °C in a LEEC anhydric incubator in a humidified atmosphere consisting of 95% air/ 5% CO_2 (v/v). Cells were visually assessed on a daily basis for evidence for microbial contamination.

B16 cells were grown as a monolayer cultured in MEM and subcultured at 80-100% confluence. Cells were subcultured 1in 10 i.e. 175cm² flasks were rinsed 3x with 10ml aliquots of PBS and then incubated at 37 °C with 2ml of a 0.02% EDTA solution for approximately 5 minutes to detach the cells from the flask. The detached cells were diluted to 10ml with MEM, counted on the haemocytometer to calculate concentration, and an appropriate aliquot of suspension transferred to a sterile 175 cm² flask containing 50 ml of fresh culture medium. For routine culture, cells were subcultured 1 in 10 twice weekly and the medium changed every 48 hours.

2.2.5.4.3. Determination of cell concentration

After detachment of cells, the suspension was briefly vortexed to ensure uniformity. 100 µl of the suspension was taken and to it was added 200 µl of 0.1% w/v trypan blue solution. A few drops of the samples were loaded onto a grid haemocytometer overlaid with a cover slip. The viable cell number in the four squares surrounding the central square was counted and this was repeated for the other side of the haemocytometer grid using an inverted light microscope. Viable cells were detected

by a bright 'halo'of light around their cell membrane, where as dead cells took up the dye.

The following equation was used to calculate cell concentration:

$$\text{cells/ml} = \frac{\text{Total cell count in 8 chambers} \times 10^4 \times 5}{8}$$

2.2.5.4.4. Cell Storage and Recovery

Cell suspensions were prepared from a confluent monolayer during subculture and centrifuged at 1000 rpm for 8 minutes. The supernatant was then discarded and the cells resuspended in a filter sterilised (0.2 μm Acrodisc) solution of culture medium supplemented with 10% DMSO as a cryoprotectant. Cell suspensions were then dispersed in 2.0ml aliquots in Corning tubes, transferred to a Union Carbide BF6 biological freezer unit plug which fitted into a Union Carbide LR-40 liquid nitrogen freezer. Left to cool down to -70 ° C at a rate of 1 ° C/ min. The cells were then transferred to a Union Carbide LR-40 liquid nitrogen unit for long term storage.

For cell recovery, the contents of an ampoule were rapidly thawed by brief incubation at 37 ° C in a water bath. Then diluted with 10ml of fresh MEM, added over approximately 2 minutes. Cells were centrifuged for 8 minutes at 1000 rpm and the supernatant was discarded. The cells were then transferred to a 175 cm^2 flask with 50 ml of MEM and incubated under standard conditions. Before any experiments were carried out the cells were cultured for at least two passages.

2.2.6. Transfection of Mammalian Cells

2.2.6.1. Preparation of Transfection Complexes

For freshly prepared controls, 2 μg plasmid DNA (pRSVβ-gal) was diluted to 250 μl with HBS in a sterile polystyrene tube (Costar, UK). Quantities of the GDV that would give charge ratios of 2.4 were separately diluted to 250 μl with HBS. Complexes were prepared by adding the 250 μl of GDV to the 250 μl of DNA. Gentle mixing of the two solutions was executed by pipetting the solution up and down 5 times using a 1 ml Gilson pipette. The spray dried plasmid DNA/liposome complexes were re-hydrated with sterile milli-Q water and an appropriate volume was taken to give 2 μg DNA per well and diluted up to 500 μl with HBS. All complexes were incubated for 15 minutes at 37 °C.

2.2.6.2. Transfection of Cells

B16 cells were seeded at a density of 10^5 cells per well on a 6 well plate and incubated for 16-20 hours under standard condition. One hour prior to transfection, the monolayers of rapidly dividing cells were washed with warm Opti-MEM^{TM}, which is a serum-free medium specifically designed for transfection experiments and then 1.5 ml of this medium was added to each well. Five hundred microlitres of freshly prepared complexes containing 2 μg of DNA and the rehydrated spray-dried plasmid DNA/ liposome complexes were then added to the culture medium overlaying the cells. Cells were transfected for 4 hours at 37 °C after which time the transfection medium was replaced with fresh culture medium and cells were cultured for a further 44 hours before

harvesting for analysis. For all transfection studies, each data point represents the mean ± standard error of the mean (SEM) of triplicate samples and each experiment was repeated 3 times.

2.2.7. Methods for Quantifying Expression

2.2.7.1. Preparation of Cell Extracts for Analysis

Solutions

Phosphate buffered saline (PBS)

Lysis buffer (0.1% [w/v] Triton X - 100 in 250mM Tris buffer pH 8)

2.2.7.2. Detergent Lysis Method

After the appropriate period of incubation post transfection the cell monolayer was washed twice with 2 ml of PBS. Cells were then treated with 250 µl of lysis buffer per well and the plates were then frozen at -70 ° C. After thawing at room temperature for 30 minutes, the cell extracts were collected and transferred into sterile micro-centrifuge tubes and centrifuged for 10 minutes at 1000 rpm and the supernatant used for analysis.

2.2.7.3. o-nitro-phenyl-ß-D-galactosidase (ONPG) (MacGregor *et al*, 1991)

Solutions

2x ONPG assay buffer 1.35 mg/ml ONPG
2 mM Mg Cl_2
40 mM 2-mercaptoethanol
100 mM sodium phosphate (pH 7.3)

1M di-sodium carbonate

0.1 M sodium phophate buffer (pH 7.3)

An appropriate volume of cell extract (maximum of 100 µl) was diluted to 300 µl with 0.1M sodium phosphate buffer. This was incubated for 10 minutes at 37 °C and 300 µl of 2x assay buffer previously incubated at the same temperature was added. The mixture was briefly vortexed and incubated for a further 30 minutes. After the 30 minutes the reaction was terminated by the addition of 1 ml of 1M di-sodium carbonate solution. The production of σ-nitro-phenol, a yellow chromogenic substance liberated by hydrolysis of ONPG, was measured at 420nm on a Milton Roy 750 spectrophotometer. A typical standard curve is shown in appendix C1.

2.2.7.4. Protein Assay

β-galactosidase activity was converted to relative values by dividing the assay per well by the amount of protein (mg) in the well at the end of the transfection. The protein content in cell extracts was measured using the Bio-Rad Dc protein assay kit. Samples were prepared according to the manufacturer's protocol and calibration curves were constructed using bovine serum albumin as a standard. Spectrophotometric measurements were made at 750nm. A typical standard curve is shown in appendix C3.

2.2.7.5. Calculations

2.2.7.5.1. Charge ratios

$$\text{Number of moles of positive charges} = \frac{\text{Mass of GDV} \times \text{Number of positive charged groups}}{\text{Molecular weight of GDV}}$$

$$\text{Number of moles of negative charges} = \frac{\text{Mass of DNA}}{330}$$

$$\pm \text{ Charge ratio} = \frac{\text{Number of moles of positive charges}}{\text{Number of moles of negative charges}}$$

where 330 is the average molecular weight of a nucleotide (reviewed by Felgner *et al*, 1997).

2.2.7.5.2. Transfection Activity

$$\beta\text{-galactosidase activity per well} = \frac{250 \times \beta\text{-gal activity per sample}}{\text{sample volume } (\mu l)}$$

$$\text{Protein content per well} = \frac{250\text{x protein content per sample}}{\text{sample volume } (\mu l)}$$

$$\text{Transfection efficiency} = \frac{\text{Total } \beta\text{-gal activity per plate (mU or RLU)}}{\text{Total protein per plate } (\mu g)}$$

where RLU is relative light units and mU is the activity of β-galactosidase in milli units.

2.3. RESULTS

2.3.1. Spray Drying of the Lipoplexes

Spray drying was achieved using the Research Scale Spray Dryer and adopting the 'open cycle' process. The conditions were documented in the tables below. As shown in Table 2.2a masses of 400 μg per batch were spray-dried. The tables below also show that the conditions during spray drying of all batches were controlled quite rigorously. One of the most important parameters regarding spray drying is the outlet temperature, which can greatly influence the morphology of the final product. This remained fairly constant throughout the spray drying cycle, between a range of 72.8-84.6 °C over the four batches. However, the percentage recovery was low, which indicated there was a lot of room for improvement in the spray drying process itself. The percentage of mass recovered over the four batches spray-dried was 35.25-43.75%.

Tables 2.2 a-d Spray Drying Conditions

(a) Feed Material:-

Conditions	Control Formulation 1	Formulation 1	Control Formulation 2	Formulation 2
Material Type	*DOTAP/ Mannitol*	*DNA/DOTAP/ Mannitol*	*DOTAP:DOPE/ Mannitol*	*DNA/DOTAP: DOPE/Mannitol*
Total Solute Concentration (%w/v)	10	10	10	10
Volume (ml)	4	4	4	4
Mass Spray Dried (g)	0.4	0.4	0.4	0.4

(b) Feed Details: -

Conditions	Control Formulation 1	Formulation 1	Control Formulation 2	Formulation 2
Pump Settings	95 rpm	95 rpm	95 rpm	95 rpm
Tube Detail	0.5D/1.6D	0.5D/1.6D	0.5D/1.6D	0.5D/1.6D
Feed Rate (g/min)	*Batch 1/Batch 2* 0.70/0.72	*Batch 1/Batch 2* 0.72/0.72	*Batch 1/Batch 2* 0.74/0.72	*Batch 1/Batch 2* 0.70/0.76

(c) Drying Conditions: -

Conditions		Control Formulation 1		Formulation 1		Control Formulation 2		Formulation 2	
Mini Spray Dryer		*Batch 1*	*Batch 2*	*Batch 1*	*Batch 2*	*Batch 1*	*Batch 2*	*Batch 1*	*Batch 2*
Inlet Temperature (°C)		130		130		130		130	
Outlet Temperature (°C)	Start	82.0	75.8	76.9	72.8	73.8	76.8	77.9	76.9
	End	84.6	73.5	78.0	73.1	81.1	82.7	79.6	79.0
Atomisation Type		Twin-fluid		Twin-fluid		Twin-fluid		Twin-fluid	
Atomisation Pressure (barg)		3.0		3.0		3.0		3.0	
Atomisation Airflow (l/min)		23.0	22.0	20.0	22.0	22.0	22.0	21.0	21.0
Drying Air Pressure (barg)		1.0		1.0		1.0		1.0	
Drying Airflow Rate (l/min)		5.0		5.0		5.0		5.0	

(d) Recovery: -

	Control Formulation 1		Formulation 1		Control Formulation 2		Formulation 2	
Mini Spray Dryer	*Batch 1*	*Batch 2*	*Batch 1*	*Batch 2*	*Batch 1*	*Batch 2*	*Batch 1*	*Batch 2*
Mass of Vessel (g)	22.719	23.388	22.931	22.749	22.350	23.028	23.426	23.248
Mass of Vessel+Product (g)	22.883	23.552	23.106	22.900	22.491	23.189	23.584	23.406
Mass Recovered (g)	0.164	0.164	0.175	0.151	0.141	0.161	0.158	0.158
Recovery (%)	41.0	41.0	43.75	37.75	35.25	40.25	39.5	39.5

2.3.2. Microscopic Imaging of the Spray Dried Microparticles

The morphology of the spray dried formulations of lipoplexes were examined using light microscopy at a magnification of x400. Typical images of the spray dried lipoplex microparticles are shown in Figure 2.2. The microparticles from both batches appear to have smooth surfaces with some particles containing internal voidages i.e. they appear toroidal in shape. Although microscopic studies suggested that particles were aggregated it was thought that this was due to the difficulty of dispersing particles in acetone on microscope slide, as the acetone evaporated off quickly. For sizing studies the particles were sonicated in acetone, before sizing in isobutyl alcohol, which gave rise to a more homogenous dispersion.

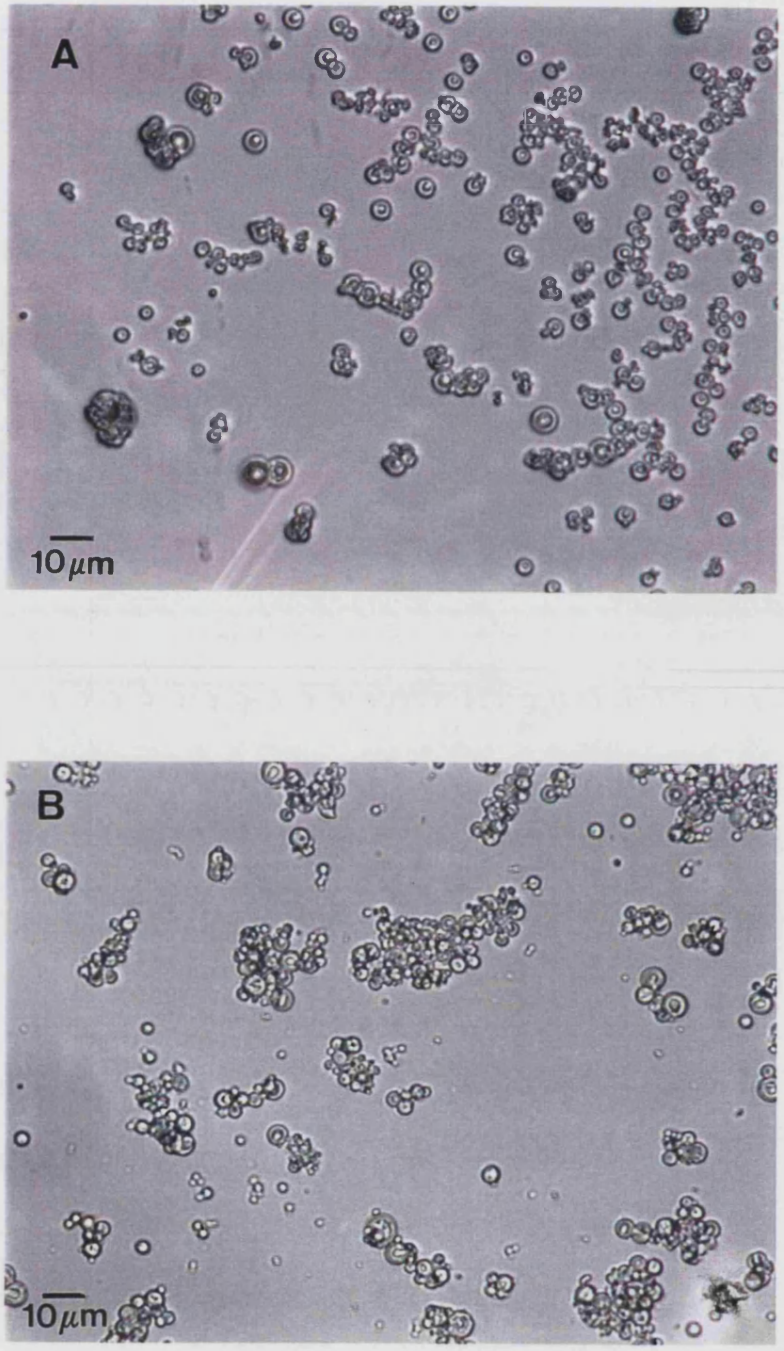
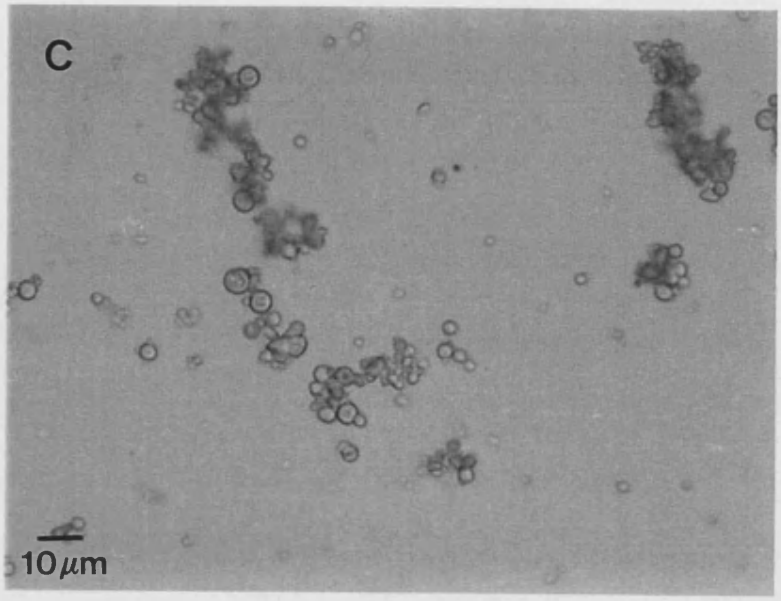


Figure 2.2a Light microscopy images showing morphology of the spray dried microparticles. (A) Image of microparticles produced from spray drying Control Formulation 1 (x 400). (B) Image of microparticles produced from spray drying Control Formulation 2 (x 400).



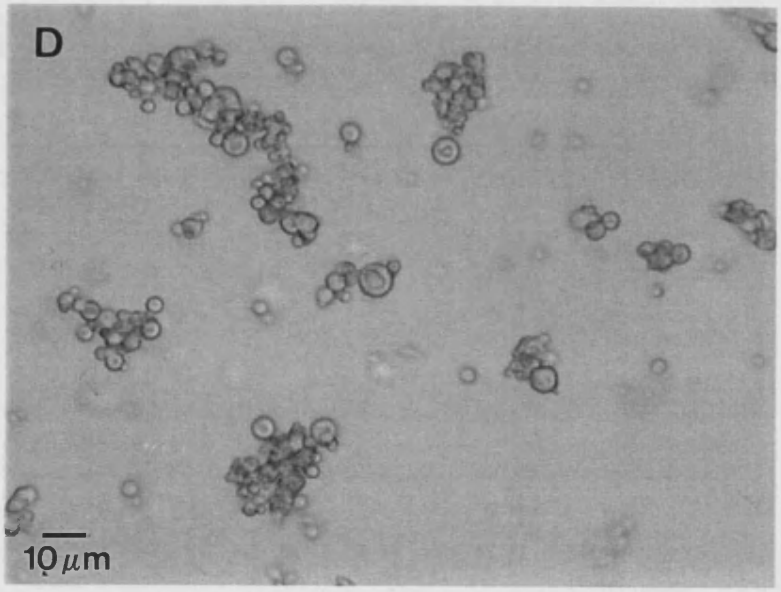
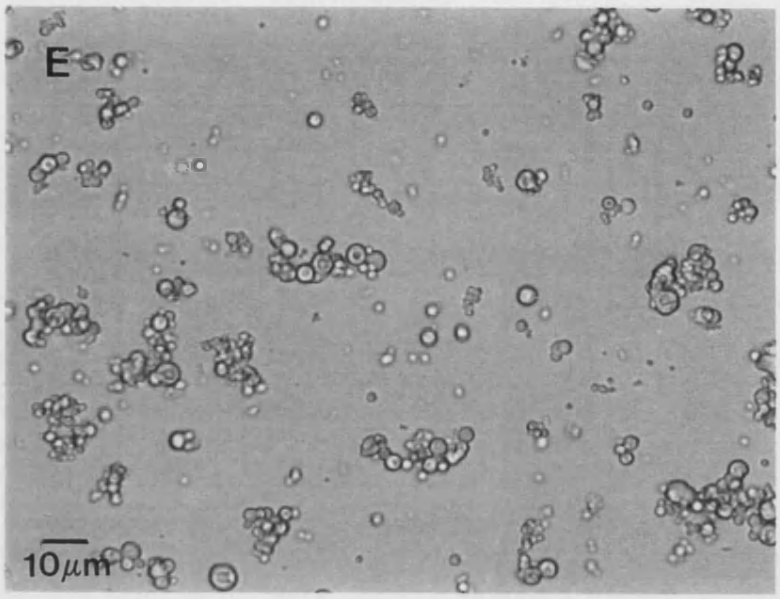


Figure 2.2b Light microscopy images showing morphology of the spray dried microparticles. (C) and (D) are images of microparticles produced from spray drying Formulation 1 (x 400).



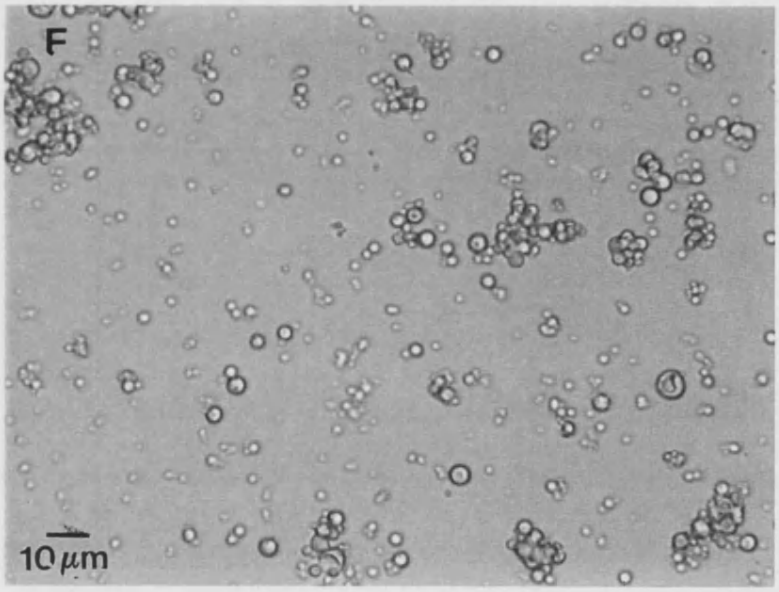


Figure 2.2c Light microscopy images showing morphology of the spray dried microparticles. (E) and (F) are images of microparticles produced from spray drying Formulation 2 (x 400).

2.3.3. Particle Size Analysis

Particle size analysis of the spray dried batches of the microparticles was achieved using the Small Volume Module Laser Sizer LS23. The microparticles were dispersed in isobutyl alcohol and background readings were taken before actual measurements of the microparticles were taken. The data below shows the volume median diameter values for the samples analysed. The values at 10, 50, and 90% levels represent the percentage cumulative undersize for the samples tested. Therefore, the value quoted at 10 % indicates that 10 % of the particles are smaller than that value and 90 % are greater than that value in the volume analysed.

Table 2.3 Mean particle size of the spray dried batches of various formulations.

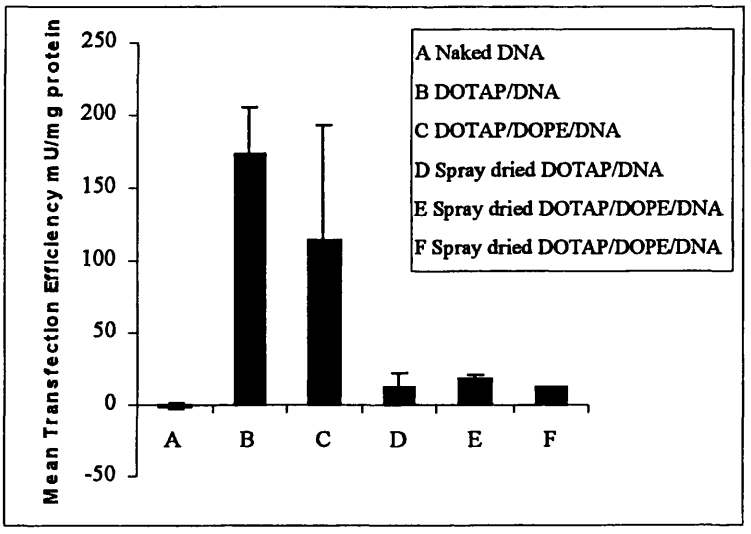
		Mean Particle Size (μm)		
Sample ID	Batch	D(0.1)	D(0.5)	D(0.9)
Control Formulation 1	1	1.419 (±1.9)	3.906 (±2.6)	9.525 (±7.6)
	2	1.338 (±5.3)	3.984 (±9.9)	9.588 (±16.7)
Formulation 1	1	1.433 (±1.0)	5.510 (±2.7)	13.10 (±4.9)
	2	1.445 (±2.9)	3.210 (±1.8)	11.48 (±3.8)
Control Formulation 2	1	4.482 (±12.0)	3.598 (±3.0)	8.470 (±4.9)
	2	5.102 (±9.0)	3.939 (±4.7)	9.250 (±14.5)
Formulation 2	*1	1.445 (±5.5)	6.02 (±7.6)	12.265 (±4.6)
	†2	1.297	4.679	10.06

* for batch 1 values represented the range of two samples. † for batch 2 values represent one sample analysed.

2.3.4 Transfection Activity of the Spray Dried Lipoplexes

The survival of the plasmid lipid complexes after the spray drying process was assessed using a bioassay. B16 cells were transfected as described in section 2.2.6. with reconstituted spray dried lipoplexes, each dose containing 2 μg DNA /10 μg lipid, assuming the materials were homogeneous.

a)



b)

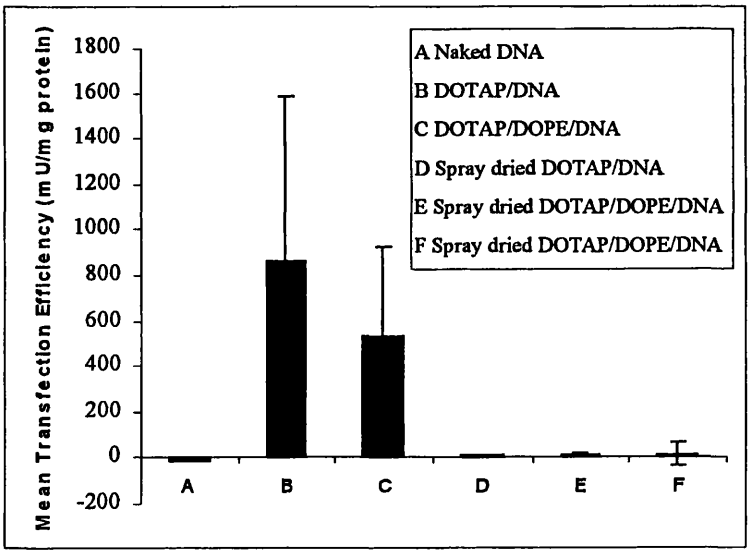


Figure 2.3 a & b Biological activity remaining after spray drying lipoplexes. B16 cells were transfected with the spray dried lipoplexes for 4hours and cells harvested after a further 44 hours for analysis of β-galactosidase activity. Data represent the mean of triplicate sample ± SEM.

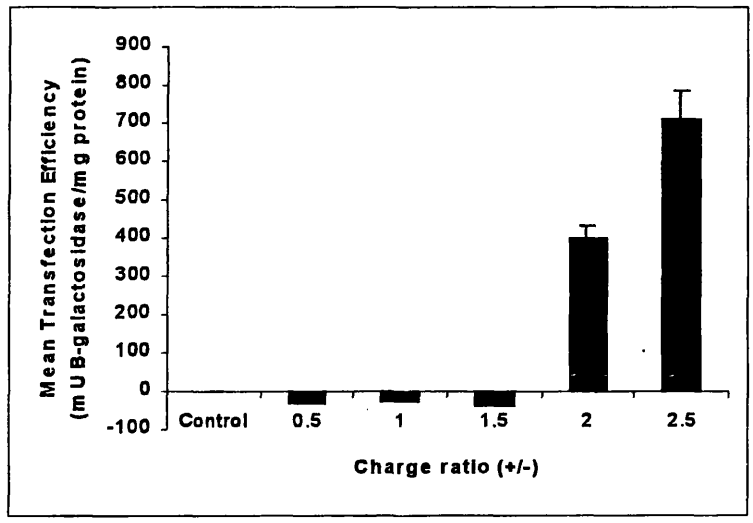
In the first experiment, shown in Figure 2.3 it appeared that some of the plasmid had survived the spray drying process, as there appeared to be some β-galactosidase activity. However β-galactosidase expression was low compared to the freshly prepared positive controls.

No attempts were made to optimise the storage conditions in these preliminary experiments so it was not clear whether loss of activity occurred during spray-drying or during subsequent storage. All batches were spray-dried and collected under aseptic conditions. The samples were stored in sealed clear vials at 4 °C, and were used for transfection approximately four weeks after preparation. The distance between the manufacturing site and testing site created problems which needed to be more carefully managed in future experiments.

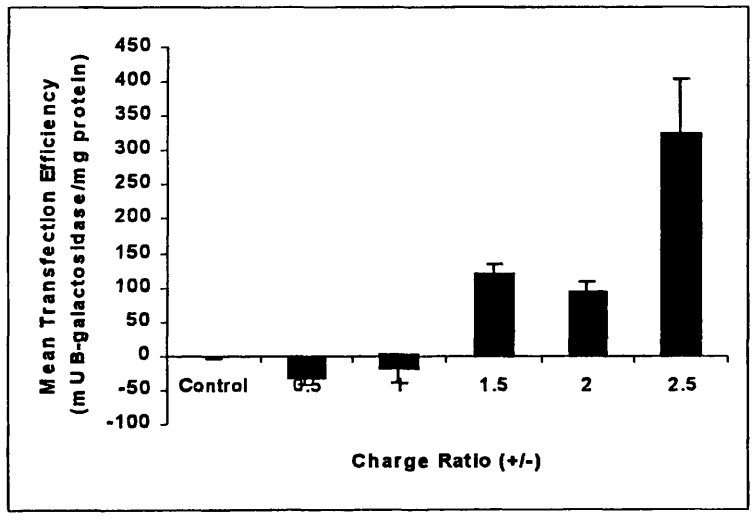
2.3.5. Optimisation of Formulation 1 for *In Vitro* Transfection

To determine the optimum charge ratio of the pCMV*lacZ*/DOTAP complex for use in future transfection efficiency experiments in B16 cells, 2 μg of pCMV*lacZ* was complexed with variable quantities of DOTAP and these freshly prepared complexes were used to transfect B16 cells for 4 hours.

a)



b)



c)

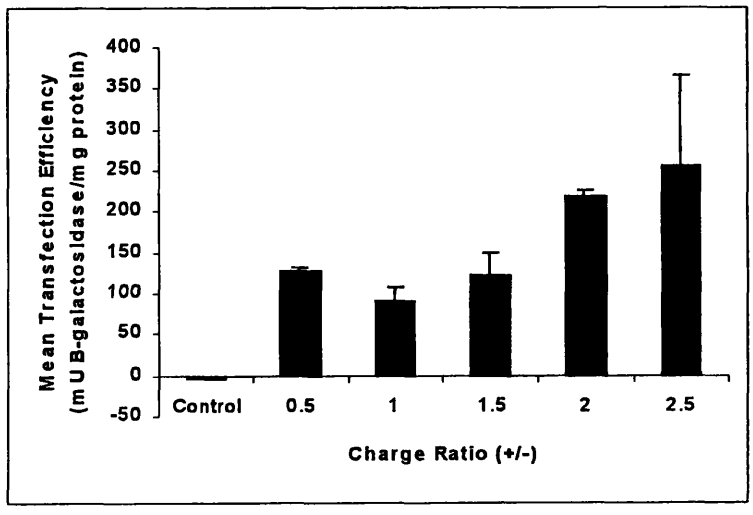


Figure 2.4 a, b & c Transfection of B16 cells with DOTAP over a range of charge ratios. B16 cells were transfected with 2 μg of pCMV*lacZ* complexed with variable quantities of DOTAP for 4hours and cells harvested after a further 44 hours for analysis of β-galactosidase activity. Data represent the mean of triplicate sample ± SEM.

As shown in Figure 2.4 it appeared that to achieve a high level of expression the charge ratio for pCMV*lacZ*/DOTAP complexes needed to be in excess of 1.5 (+/-). Since activity has been reported to be reduced at higher charge ratios (+/-), it was considered that 2.5 (+/-) would be appropriate for future work (Uduehi, 1997).

2.3.6. Homogeneity of Spray Dried Formulation 1

To determine whether formulation 1 was homogeneous after spray drying a single batch was spray dried and aliquoted out into hplc vials. Samples were taken reconstituted within 24 hours and used to transfect B16 cells for 4 hours using lipoplexes at a charge ratio of 2.5.

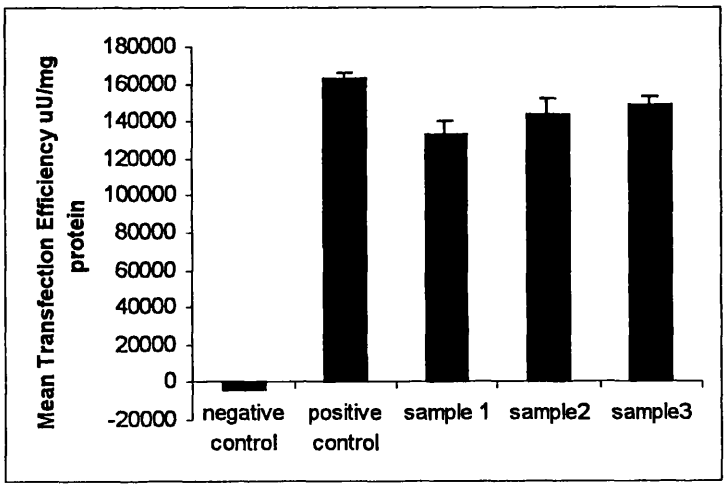


Figure 2.5 Biological activity remaining in samples after spray drying lipoplexes. B16 cells were transfected for 4hours with the spray dried lipoplexes within 24hours of preparation for 4hours and cells harvested after a further 44 hours for analysis of β-galactosidase activity. Data represent the mean of triplicate sample ±SEM.

As shown in Figure 2.5 it appears that there seems to be no apparent difference between samples. A relatively small amount of activity (an average of 13.1%) was lost during the spray drying process.

The integrity of the plasmid was also examined by running spray-dried samples an on a 0.8% agarose gel. Traces of the plasmid could be observed. Only one band was evident, which corresponded to the single band obtained from the DNA from the original stock. Supercoiled DNA was generally represented at a proportion of about 70 –80 % of the total in common with the starting material. The expectation is that the supercoiled DNA is the active material since strand nicks were likely to be random.

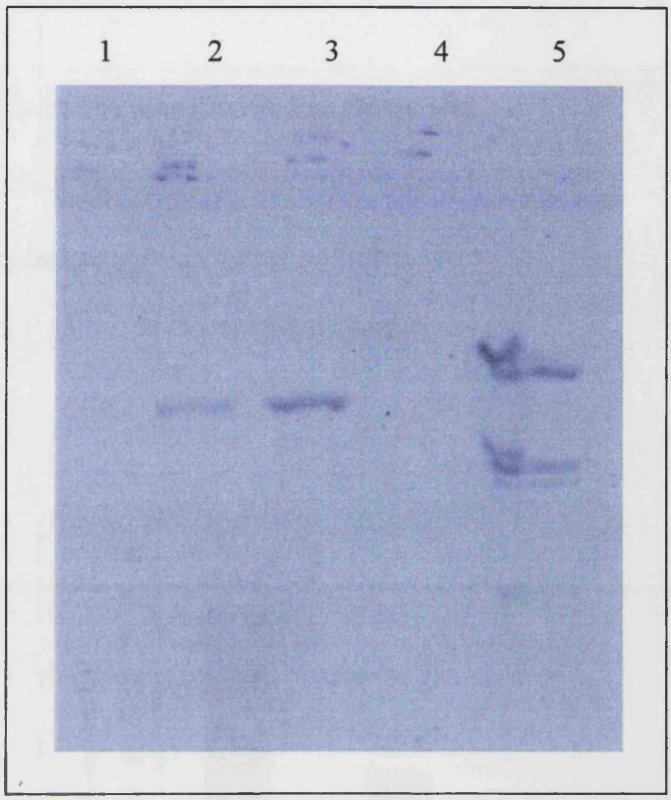


Figure 2.6. Agarose Gel showing the presence of plasmid DNA in the Spray Dried Complex (see section 2.2.4.3.). Lane 5 contains *Eco*R I/Hind III cut $_{\lambda}$DNA marker. Lane 3 contains pCMV*lacZ* from original stock. Lane 2 contains spray dried pCMV*lacZ*. Analysis of plasmids run on a 0.8% agarose gel for 1 hour and 45 minutes at 80V.

2.3.6. Stability of Spray Dried Lipoplexes

The stability of the spray-dried lipoplexes was evaluated after storage at room temperature. Stability was assessed by percentage of β-galactosidase remaining after incubation at room temperature in amber coloured vials. Samples were taken from vials packaged aseptically and used to transfect B16 cells at intervals of 0, 1, 2, 4 weeks.

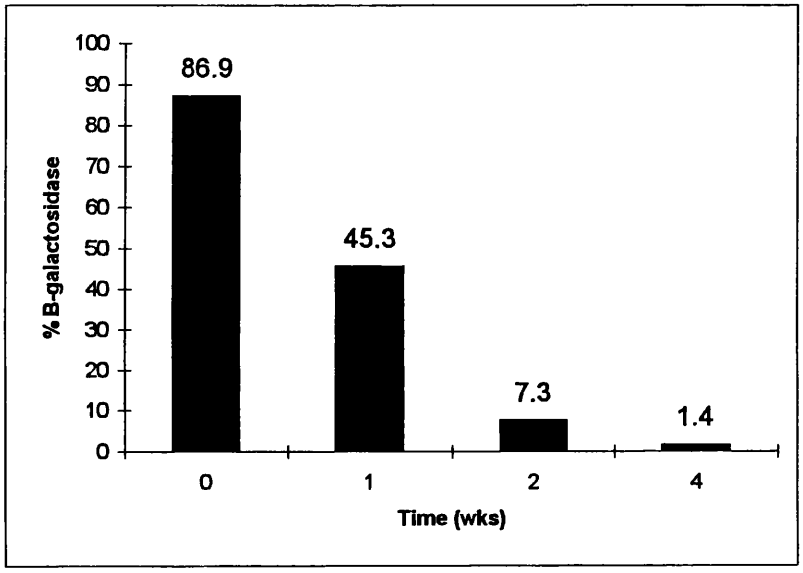


Figure 2.7 Percentage of β-galactosidase remaining after incubation at room temperature. B16 cells were transfected with the spray dried lipoplexes over a period of 4 weeks. Transfection was for 4hours and cells harvested after a further 44 hours for analysis of β-galactosidase activity. Data represent the mean of triplicate sample ± SEM.

Figure 2.7 shows that the spray dried lipoplexes were not very stable at room temperature as β-galactosidase activity seems to have decreased by practically half after the 1st week and very rapidly after that. This confirmed earlier results indicating a loss of activity after four weeks (Figure 2.3).

2.4. DISCUSSION

Experiments described in chapter 2 were designed to assess the use of soluble microparticles, produced by adopting Quadrant's proprietary spray drying process, which produces microparticles from 0.1 to 50 microns, in gene therapy.

All transfection results presented in this thesis represent data from a single experiment although all the experiments were reproduced in triplicate. The decision to show a representative experiment instead of the average of the triplicate experiments was made for two reasons, firstly taking the mean of triplicate experiments would not allow intra-experimental variability to be easily assessed and secondly, the specific β-galactosidase activity was found to vary between experiments. Although the rank order of the various factors within each study was generally consistent. Inter-experiment variability is a well recognised problem in transfection studies and is commonly observed in biological systems (Boussif *et al*, 1996). This variability was due to various factors such as environmental conditions, cell passage number and the growth rate of cells, that most of which are not readily controllable.

Before any spray drying could be done a formulation had to be proposed. Mannitol, a sugar was used as the carrier material for its protectant properties (Broadhead, 1994). It had all ready been established at Quadrant that it was possible to spray dry mannitol at low temperature and form consistently size controlled particles as hollow spheres.

pRSV*lacZ* was chosen as a reporter gene because of the versatility in methods currently available for detecting the gene product, β-galactosidase. This is a bacterial enzyme derived from *Ecoli*, which cleaves β-galactosides into their constituent sugars. The relatively high levels of enzyme activity in B16 cell extracts following transfection

with pRSV*lacZ* meant that quantification by UV absorbance measurements using σ-nitro-phenyl-β-D-galactoside (ONPG) was possible. In order to make reasonable comparisons between and within experiments, a suitable method for calculating transfection efficiency had to be adopted. One option was to examine the total enzyme activity (mU β-galactosidase) in cell extracts or standardise the results by taking into account the protein content in corresponding samples i.e. specific β-galactosidase activity (β-galactosidase/mg protein). The protein content in all extracts is directly proportional to the viable cell number. Hence the latter approach was used because it accounted for cell viability, an important factor when considering the toxicity of the different gene delivery vectors.

The cationic lipid DOTAP was used as it is already a well-established gene delivery vector, and is known to give high levels of transfection efficiency in B16 cells (Uduehi, 1997). The neutral lipid DOPE was used, as it has been reported to be successful in transfection experiments in conjunction with some cationic lipids, promoting formation of stable liposomes, and is believed to enhance the cytoplasmic delivery of DNA (Felgner *et al*, 1994).

The conditions of the spray drying process were well controlled. The outlet spray drying temperatures were in the range of 70°-85° C. The slight variation in temperature is a batch to batch difference due to differing days. The percentage recovery of the product was 35.25-43.75% which appears to be low, although this is a major problem often encountered with laboratory-scale spray dryers (Broadhead, 1994). For this particular method and taking into account however that only 10 % w/v was spray dried this was quite a good level of product recovery. Also the feed rate and the inlet temperature and the interaction between the two determines the yield obtained

(Broadhead, 1994) e.g. the lowest yield was observed when the feed rate was at its highest (however, in this case the inlet temperature is fixed).

As can be seen from the photographs all the formulations formed spherical hollow microparticles under the spray drying conditions used. This phenomenon is governed partly by the conditions used during the spray drying process, in particular the relationship between inlet temperature and the boiling point of the solvent, and partly down to the chemical nature of the solute. Drying of the droplets can be divided into 2 phases the 1st phase being generally similar in most cases as it is independent of the chemical nature of the solute. As the droplet forms rapid evaporation transpires at the surface, thereby introducing concentration gradients between the liquid surface and interior, the former becoming more concentrated in solute, this eventually leads to "crust" formation. From this point the shape of the final particle is dependent on the porosity and rheology of the crust i.e. the chemical nature of the solute, plus the 2nd drying phase i.e. it is here that the relation between inlet temperature (drying temperature) and the boiling point of the solvent are most significant. If the inlet temperature is below the solution boiling point, then three cases can be delineated, depending on material properties. For porous crusts, further evaporation occurs, although at a less rapid rate than in the first period, by diffusion of solvent through the pores to the interface accompanied by no change in the particle size for rigid materials. For less porous solid skins, stresses develop that fracture the crust followed by evaporation as in the first period. Materials exhibiting viscoelasticity undergo shrivelling as the particle volume decreases during evaporation, producing a "raisin" like morphology.

Alternatively, if the inlet temperature is higher than the boiling point then as before no change in particle size occurs for porous solid skins. For more impervious,

rigid crusts, the decreased evaporation rate after crust formation results in a rise of the encased solution temperature to boiling point, and subsequent bubbling results in minor or major fractures presumably depending on the drying temperature and extent of void space available for solvent diffusion through the solid surface structure. In contrast, for low porosity viscoelastic crusts, the positive pressure can cause inflation to sizes larger than the droplet diameter, which can be followed by collapse if the skin permeability increases to sufficiently large value upon expansion. If the solid skin permeability does not increase significantly ruptures can develop, leading to shrivelling and in some cases a "spongy" structure if multiple inflation/collapse events occur.

Therefore once the crust has been formed the chemical nature of mannitol probably gave it rigid and porous properties, and the inlet temperature being at 130°C and the boiling point of water being only 100° C lead to the spherical hollow microparticles being formed (Sacchetti & Van Oort, 1996).

The most important step in successfully spray drying a drug for respiratory therapy is to control the particle size distribution. As these particles were for pulmonary delivery in the long term, hence the use of the pneumatic atomiser as they can be readily adapted to inhalation drugs or excipients both in bench-scale and production scale work, as has been demonstrated by scientific literature and patents. (Sacchetti & Van Oort, 1996).

The fundamental aspects of the spray drying process control the particle size: droplet formation, solvent type, solution concentration and droplet evaporation dynamics. All of the latter were discussed earlier except for droplet formation, which is affected by the type of atomiser used. There are many variations of the pneumatic nozzle, but we will be concentrating on the use of the external mixing nozzle, as it is the one applied to this thesis. The liquid feed is usually pumped at relatively low flow rates

through a tube and at the discharge orifice is impinged by a gas under pressure. The velocity of the gas is much larger than that of the liquid at the orifice, and it is this relative velocity that provides the force and work needed to create the surface area of a spray. In practise these two variables are controlled by the flow rate of the liquid and the gas pressure for a given nozzle.

On the fine end, sprays with a mean droplet size of 5-20 μm can be produced at high relative velocity and the reverse for coarser particles.

As can be seen in Table 2.3 generally the particle size distribution at 50% cumulative under size for all four batches fell in the range required for deposition of particle in the airways, 3.210-6.02 μm. An interesting point was that with incorporation of the plasmid the volume median diameters of the spray-dried microparticles increased.

The integrity of the plasmid was assessed post spray drying, as plasmid DNA is a sensitive material and may be prone to thermal degradation at high temperatures. Figure 2.3 shows that there was β-galactosidase activity present although it appeared to be quite low. The latter may be accounted for by the fact that these transfection experiments were not carried out immediately after the batch was spray dried but it was at least 4 weeks, due to the time taken to set up the initial tissue culture experiments. Further experiments were required to assess the stability of the product.

Other factors which might have also contributed to the low enzyme activity are that the B16 cells were confluent at the time of transfection, which has been reported to result in a lower level of transgene expression (Vitiello *et al*, 1996). Distribution of the plasmid throughout the spray-dried batch, and the net charge of the complex formed with DNA (Fasbender *et al*, 1995), which has been reported to affect transfection efficiency levels.

Before another batch was prepared to investigate the formulation in terms of transfection efficiency it was necessary look at the optimum charge ratio of plasmid DNA (pCMV*lacZ*). Figure 2.4 shows that an increase in the quantity of DOTAP led to an increase in transfection activity. The optimum charge ratio in the range tested of 0.5 – 2.5, was found to be 2.5. Charge ratios higher than 2.5 in this case were not assessed as they have been reported to be toxic *in vivo* (Lasic & Templeton, 1996), which would not be productive if this formulation was tested *in vivo*.

The homogeneity of the spray-dried batch was assessed via transfection of B16 cells. The spray-dried batch was aliquoted out into amber coloured small HPLC vials, and stored at room temperature. Random samples were taken and the batch was transfected within 24 hours of spray drying. Figure 2.5 shows that only a small amount of activity was lost during the process. Also the formulation appeared to be homogeneous, as there were no significant differences between the samples taken at random.

Stability of spray dried batches was assessed by the percentage of β-galactosidase remaining after incubation at room temperature. Figure 2.6 shows that the spray dried lipoplexes were not stable at room temperature as activity appeared to have decreased by half after 1 week and then continued to decrease over 4 weeks. It is not possible to explain the nature of this instability at present, but clearly this aspect of the system would need to be carefully examined if spray-dried particles are to be developed.

It has been established that cationic lipid-DNA complexes can be entrapped in spray-dried mannitol particles which are a suitable size for lung deposition. Biological activity, as assessed by transfection of mammalian cells in culture, can be recovered when the particles are dispersed in aqueous solution. However it is now known that cationic lipid-DNA complexes are relatively inefficient gene delivery systems *in vivo*,

due to their inability to deliver DNA to the nuclei of non-dividing cells. Thus we consider that the mannitol systems is a promising carrier technology for inhalation which is waiting for a more efficient intracellular DNA delivery system, specifically a system which is able to transfect differentiated, non-dividing epithelia. Given the lack of efficiency a decision was made not to examine the mechanism of instability of spray-dried lipid:DNA complexes at this stage.

CHAPTER 3

OPTIMISATION OF A LUCIFERASE REPORTER ASSAY

3.1. INTRODUCTION

Before commencing a pre-clinical study or even gene transfer to cells in culture it is essential to test the variables and pinpoint the conditions leading to the best use of the reporter gene. All the transfections described in Chapter 2 were carried out using the pRSVß or pCMVß plasmid. The ß-galactosidase protein is useful as a reporter because its half-life *in vivo* is 20 hours compared to 3 hours for the commonly used luciferase protein (Tsuneoka and Mekada, 1992). However, the sensitivity of the assay is decreased due to the relatively high levels of endogenous ß-galactosidase activity in some mammalian cells. In contrast, the luciferase protein is foreign to mammalian cells and as such gives very low background levels resulting in higher sensitivity and a lower detection limit (1 fg). Luciferase reporter genes have been used successfully for assessment of gene transfer using non-viral gene delivery vectors *in vivo* (Schwartz *et al.*, 1995; Thierry *et al.*, 1995). This chapter concentrates on the optimisation of a luciferase reporter assay for analysing future gene expression studies in B16 mouse melanoma cells.

3.2. METHODS

3.2.1. DNA Preparation

3.2.1.1. Bacterial Strain

Escherichia coli strain DH5α was used for the propagation of the plasmids.

3.2.1.2. pCMVluc

The pCMV luciferase plasmid was kindly donated by J P Behr (CNRS, Strasbourg). The plasmid map can be seen in appendix A.

3.2.1.3. pTDE-SV40

The pTDE-SV40 plasmid was kindly donated by R G Vile (ICRF, London). pTDE-SV40 is a synthetic firefly luciferase reporter gene construct, under the control of the SV40 promoter and extra enhancer elements, which are responsive to cAMP. The plasmid map can be seen in appendix A.

3.2.2. *In Vitro* Transfection of B16 to Investigate the Time-course of Luciferase Expression using pCMV*luc* and pTDE-SV40 Luciferase Reporter-Gene Systems

To follow the onset and duration of luciferase expression in B16 mouse melanoma cells when driven by the CMV promoter, transfection complexes of DOTAP/pCMV luciferase were freshly prepared at charge ratio of 2.5 and B16 cells were cultured as described in Chapter 2. After incubation of transfection complexes with B16 cells for 4 hours, the cells were washed with complete medium and incubated for a further 12, 24, 32, 40, 44, 48 and 72 hour period. The cells were then harvested for analysis, as described in Chapter 2.

The above was repeated for transfection of B16 cells with pTDE-SV40 to compare the onset and duration of expression of the luciferase reporter gene, when driven by two different promoters in the same cell line. B16 cells were analysed using the luciferase assay.

3.2.2. Stimulation of the Luciferase Reporter-Gene Systems pCMV and pTDE-SV40

To see if expression of the reporter gene in B16 cells could be enhanced further the reporter gene-system was stimulated with a cAMP-elevating agent, αMSH, or the cAMP analogue 8-bromo-cAMP. The 8-bromo-cAMP was added to the B16 cells 5 hours prior to harvesting the B16 cells for analysis. For a negative control cAMP alone was used. The B16 cells were harvested 24 hours post transfection.

Further experiments were carried out with the pTDE-SV40 reporter gene-system. Comparisons were made between 8-bromo-cAMP and α melanocyte -

stimulating hormone (αMSH), which interacts with the MSH G-protein-coupled receptor to stimulate the production of cAMP via the activation of adenylate cyclase. B16 cells were stimulated with 8-bromo-cAMP and αMSH 5 hours prior to harvesting the cells for analysis. The cells were harvested 24 hours post transfection and analysed using the luciferase assay.

3.2.4. Method for Quantifying Expression

3.2.4.1. Preparation of Cell Extract for Analysis

After the appropriate period of incubation post transfection, the cell monolayer was prepared for analysis as described in Chapter 2. The lysis protocol in chapter 2 was followed substituting the Promega lysis buffer (1x: 25 mM Tris-phosphate, pH 7.8; 2 mM DTT; 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid (CDTA); 10% glycerol; 1% Triton X-100 and BSA 1 mg/ml) for the phosphate buffer. Supplementation with 1 mg/ml BSA was used to ensure that luciferase was not lost from solution by adsorption onto container walls.

3.2.4.2. Luciferase Assay

Luciferase levels within cells were quantified using the Promega luciferase assay system (Promega, UK) according to the manufacturer's protocol. As little as 10-20 moles (0.001pg) of firefly luciferase can be measured using this kit. A standard curve of light units vs. relative enzyme concentration was produced using purified firefly luciferase from Promega. After centrifugation of the cell lysate, 20 µl of cell extract

(supernatant) collected at ambient temperature were mixed with 100 µl of luciferase assay reagent (luciferase assay substrate + 10 ml of assay buffer) again at ambient temperature. The disposable test tube containing the reaction was then immediately placed into the luminometer (TD-20/20). The instrument settings were optimised as follows: sensitivity 25.1, delay 2 seconds and the integration 10 seconds. A typical standard curve is shown in appendix C2.

Luciferase activity was converted to relative values by dividing activity per well by the amount of protein (mg) in the well as described in Chapter 2.

3.3. RESULTS

3.3.1. Onset and Duration of Expression of Luciferase

The onset and duration of expression of the luciferase reporter gene was assessed in detail. B16 cells were transfected as described in section 3.2.3 with lipoplexes of pCMV*luc* or pTDE-SV40, each formed with DOTAP, at a charge ratio of 2.5. After initial incubation of the B16 cells with lipoplexes for 4 hours, the cells were washed with complete medium and incubated further at time intervals of 4, 8, 12, 16, 24, 32, 40, 44, 48, and 72 hours post transfection. The cells were harvested for analysis for luciferase activity and protein content. The luminescent assay proved to be very sensitive. Expression of luciferase could be detected immediately after the 4 hour treatment of B16 cells with pCMV*luc*/DOTAP (see Figure 3.1). Extent of expression per well increased sharply over 24 hours and was maintained at a high level until 48 hours, after which the total expression declined, (see Figure 3.2). Total protein content per well was used as a measure of cell growth during the experiments. Growth proceeded throughout the 72 hour period of the experiment (see Figure 3.3) at which point the cultures were approaching confluence. When the luminescence data was compared to controls it was possible to calculate the mass of luciferase expression per unit mass of total cell protein, which represents the relative gene expression per cell (see Figure 3.3). Peak expression per cell occurred at 24-32 hours, after which expression per cell declined steadily over the 72 hour period.

As a comparison of the behaviour of two different reporter-gene systems for the expression of the same gene in the same cell line, B16 cells were transfected with lipoplexes of pTDE-SV40/DOTAP. Onset of expression of the luciferase reporter gene

could be observed at 12 hours. Extent of expression per well increased sharply over a 32 hour period and was maintained at a high level until 48 hours, after which the total expression declined (see Figure 3.4). Peak expression per cell appears to have occurred at 32 hours.

After the time-course for the expression of the luciferase reporter gene was established, when B16 cells were transfected with the pTDE-SV40 reporter gene system, experiments were carried out to see if an expression enhancing element could affect the time-course obtained from transfection with pTDE-SV40. B16 cells were transfected as described earlier. 8-bromo-cAMP an analog of cyclic AMP was used to stimulate cells 5 hours prior to harvesting. Once harvested cells were analysed for luciferase activity and protein content.

Figure 3.5. shows that the time-course for expression of luciferase profile appeared to follow the same pattern as before stimulation was in this experiment. Expression levels of the luciferase reporter gene appeared to be higher upon stimulation with 8-bromo-cAMP.

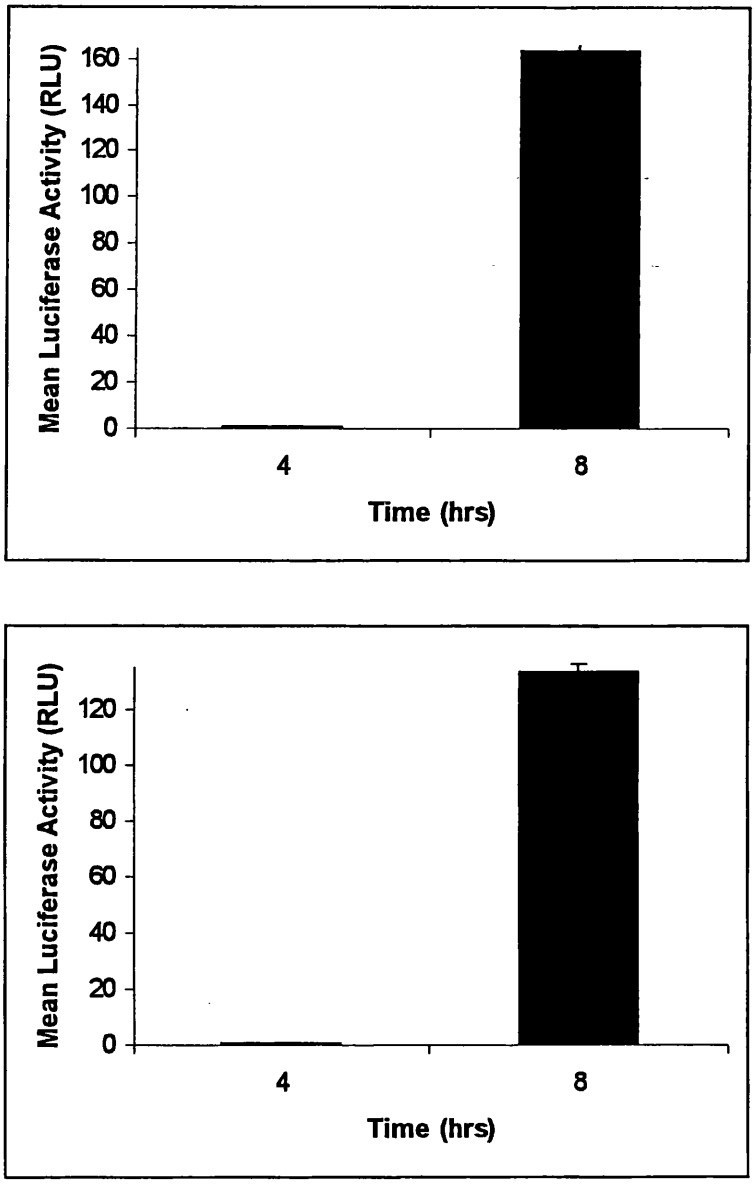


Figure 3.1. a & b. Luciferase expression after transfection of B16 murine melanoma cells with pCMV*luc*. B16 cells were incubated with the lipoplexes for 4 hours. The cells were then harvested post transfection at time intervals from 4 and 8 hours for luciferase activity. Data represents the mean of triplicate samples ±SEM..

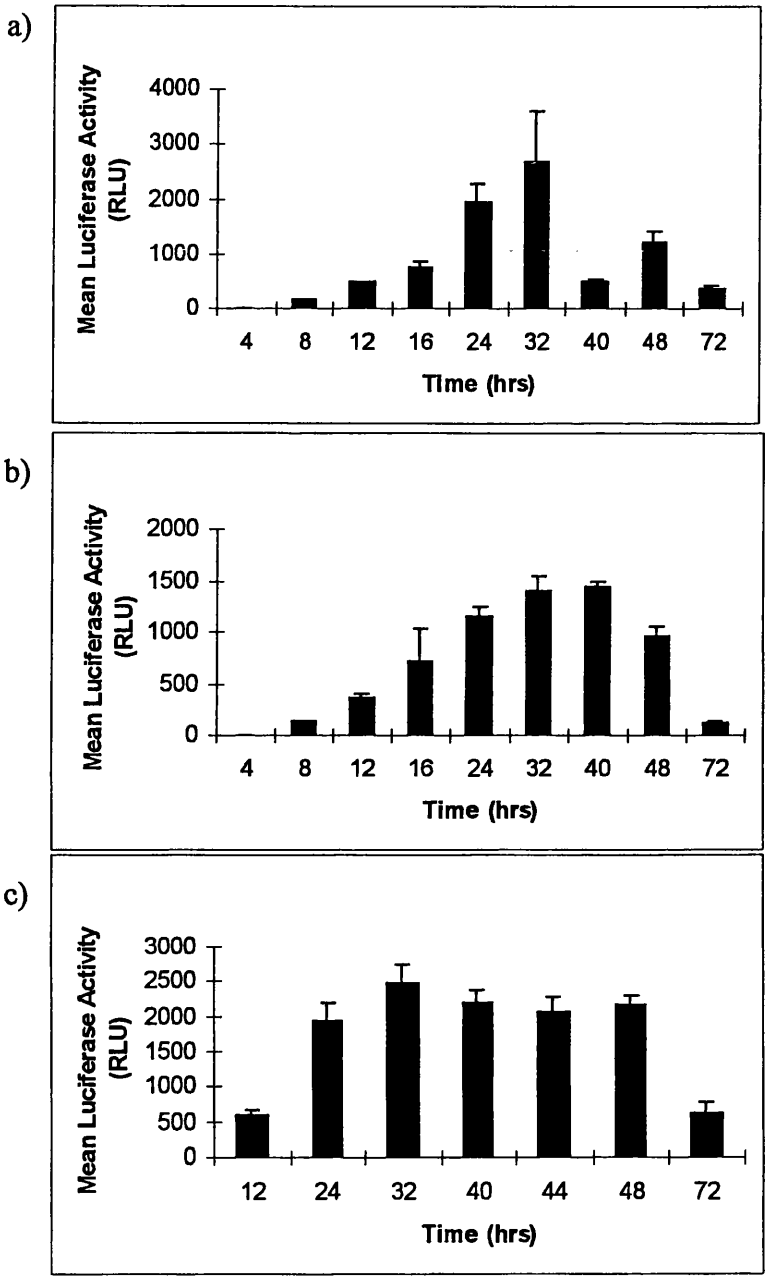


Figure 3.2 a-c. Time-course of luciferase expression after transfection of B16 murine melanoma cells with pCMV-*luc*. B16 cells were incubated with the lipoplexes for 4 hours. The cells were then harvested post transfection at time intervals from 4, 8, 12, 16, 24, 32, 40, 44, 48 and 72 hours for analysis of luciferase activity. Data represents the mean of triplicate samples ±SEM.

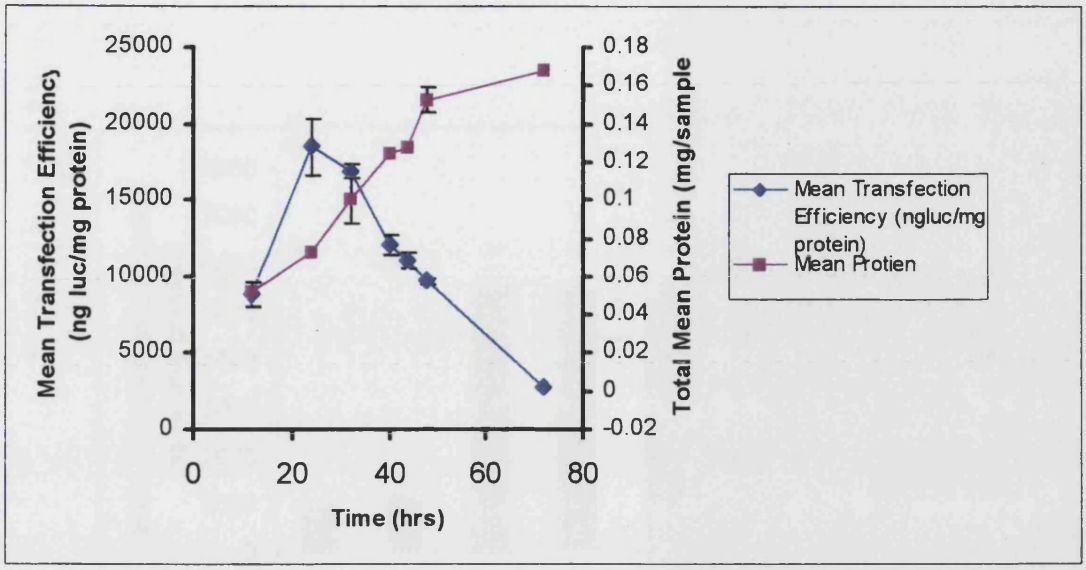
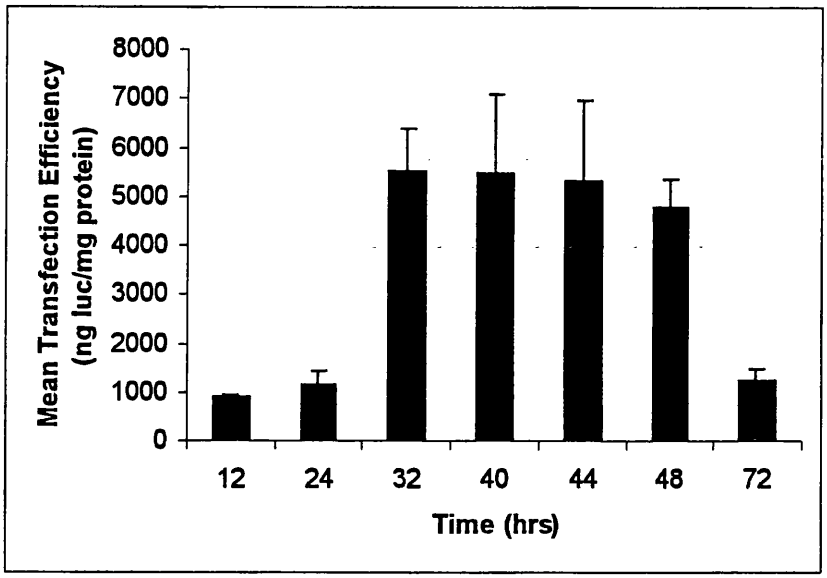


Figure 3.3. Effect of incubation period post transfection on the expression of luciferase in B16 cells transfected with pCMVluciferase/DOTAP and the total mean protein produced per sample. Data represents the mean of triplicate samples ±SEM.

a)



b)

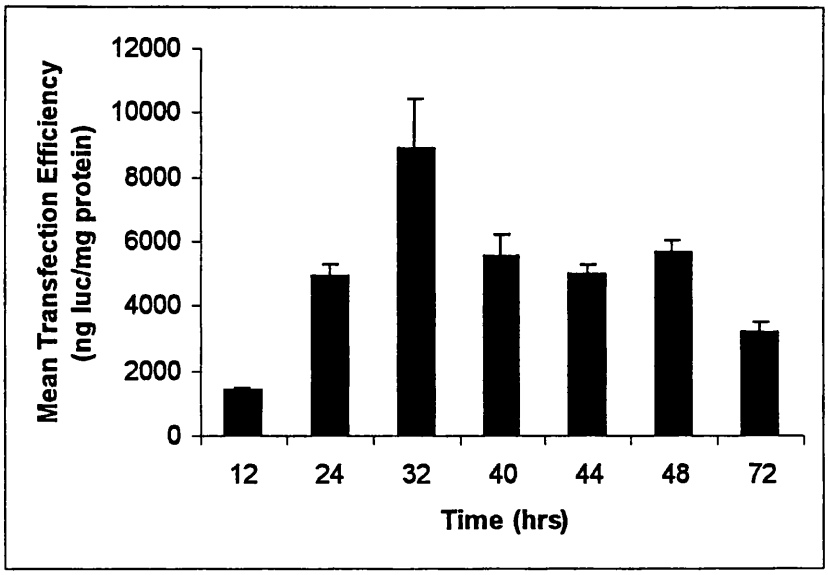


Figure 3.4 a & b. Time-course of luciferase expression after transfection of B16 murine melanoma cells with pTDE-SV40. B16 cells were incubated with the lipoplexes for 4 hours. The cells were then harvested post transfection at time intervals from 12, 24, 32, 40, 44, 48 and 72 hours for analysis of luciferase activity. Data represents the mean of triplicate samples ±SEM.

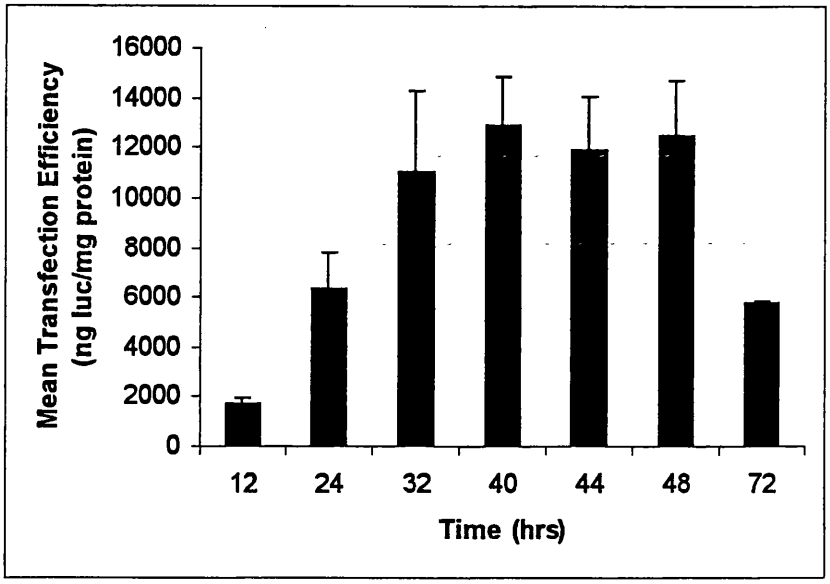


Figure 3.5. Time-course of luciferase expression after transfection with pTDE-SV40. Cells were stimulated with 8-bromo-cAMP 5 hours prior to harvesting. The lipoplexes were incubated with the B16 cells for 4 hours. The cells were then harvested post transfection at intervals of 12, 24, 32, 40, 44, 48 and 72 hours for analysis of luciferase activity. Data represents the mean of triplicate samples ±SEM.

3.3.2. Stimulation of the Luciferase Reporter-Gene Systems pCMV and pTDE-SV40

Expression of the luciferase reporter gene in B16 cells was stimulated with a cAMP-elevating agent, 8-bromo-cAMP, an analogue of cAMP. B16 cells were transfected with lipoplexes of pCMV*luc*/DOTAP and pTDE-SV40/DOTAP at charge a ratio of 2.5 as described earlier in this chapter. 8-bromo-cAMP was added to the B16 cells 5 hours prior to harvesting the cells for analysis. The B16 cells were analysed for luciferase activity and protein content. It is clear from Figure 3.6 that treatment of B16 cells with 8-bromo-cAMP after transfection with pCMV*luc*/DOTAP did not increase the expression of the luciferase reporter gene. Treatment of the B16 cells transfected with pTDE-SV40 with the 8-bromo-cAMP appeared to have induced a relatively small increase in expression of the luciferase reporter gene.

Further experiments to enhance the expression of the luciferase reporter gene were carried out using αMSH. Transfection of B16 cells with pTDE-SV40/DOTAP lipoplexes was carried out as before to compare the effects of 8-bromo-cAMP and αMSH (Figure 3.7). Stimulation of pTDE-SV40 at 24 or 48 hours did not appear to further enhance the expression of the luciferase reporter gene, although it was noticeable that the levels of gene expression were consistently higher in pTDE-SV40 transfections.

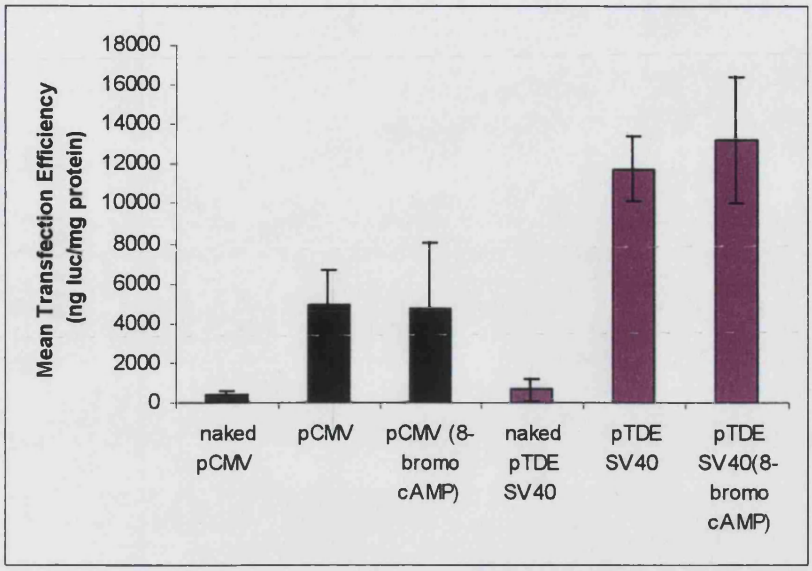


Figure 3.6. Stimulation of pCMV versus pTDE-SV40 with 8-bromo-cAMP. The lipoplexes were incubated with B16 cells for 4 hours. The cells were stimulated with 8-bromo-cAMP 5 hours prior to harvesting. The cells were then harvested 24 hours post transfection for analysis of luciferase activity. Data represents the mean of triplicate samples ±SEM.

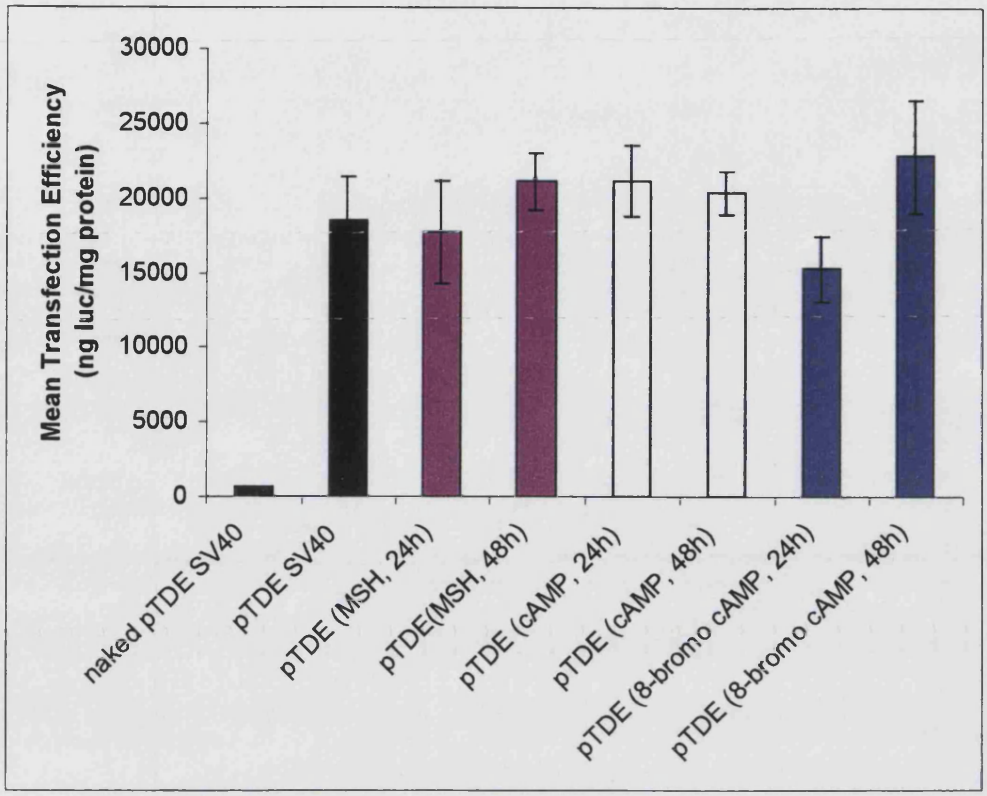


Figure 3.7. Stimulation of pTDE-SV40 with α-MSH, cAMP, and 8-bromo-cAMP, at 24 hours and 48 hours. The lipoplexes were incubated with the B16 cells for 4 hours. The cells were then stimulated with the transfection enhancers 5 hours prior to harvesting. The cells were then harvested at 24 hours and 48 hours post transfection for analysis of luciferase activity. Data represents the mean of triplicate samples ±SEM.

3.4. DISCUSSION

Expression of luciferase in B16 cells was monitored via *in vitro* transfection. Optimal expression of luciferase on transfection of B16 cells with pCMVluc/DOTAP lipoplexes was between 24-32 hours, after which expression per cell declined steadily over the 72 hour period. The decline in enzyme activity over the 72 hour period is probably due to the dilution of plasmid within the growing population of cells. Since the luciferase reporter lacks a mammalian origin of replication and remains episomal, following cell division, it will not be equally distributed to daughter cells. However, since expression *in vivo* is frequently found to be transient, there are other factors which may contribute to loss of activity. Hence, with each cycle of cell division, there were less luciferase expressing cells whilst the total protein per well increased. This suggests that the plasmid is cleared or modified.

The time for maximum gene expression was dependent on the promoter driving expression; the promoter pCMV gave maximum expression between 24-32 hours where as expression driven by the promoter/enhancer elements pTDE-SV40 was found to be at its optimal at 32 hours. However, the profile of the time-course for expression of the luciferase for both promoters was very similar onset occurred early on between 4-12 hours and decline of expression for both promoters started at 48 hour. Stimulation of both reporter gene systems with the cAMP-elevating agent α MSH or the cAMP analogue 8-bromo-cAMP was not evident (Figures 3.6 and 3.7). The B16 cells may already have had high levels of cAMP having differentiated over some time and becoming more melanotic, hence any further stimulation of the reporter genes with α MSH or 8-bromo-cAMP would not take effect. Another, possibility could be that the 5 hour treatment with these cAMP stimulating agents may be too short to allow gene expression to increase.

CHAPTER 4

CROSS-LINKED MICROPARTICLES FOR DELIVERY TO ANTIGEN PRESENTING CELLS

4.1. INTRODUCTION

Gene therapy has considerable promise in the treatment of cancer. The majority of gene therapy trials currently underway are related to cancer and most of the protocols are based on immunotherapy strategies. There are various approaches to cancer immunotherapy, one popular approach is to express genetically modified tumour vaccines. Alternatively, the environment of the tumour can be modified by expression of genes which encourage Th1 responses and induce specific cell-mediated antitumour activity (Machiewicz, 1998). A third approach is to modify the function of professional antigen-presenting cells (APCs) particularly macrophages, which have the ability to take up particles by phagocytosis. The remainder of this thesis is concerned with gene delivery to professional APCs particularly macrophages. One of the most important questions when designing gene therapy to be taken up by professional APCs is which gene delivery system best reaches the intended target cells? Particulate carrier systems such as microparticles offer promise of effective delivery of DNA or polypeptides to APCs and macrophages, since they are phagocytosed by these cells. Particulate systems have been reported to enhance the immunogenicity of DNA vaccines (O'Hagan *et al*, 1991a). The 'adjuvant effect' achieved through the association of polypeptide antigens with polymeric microparticles has also been demonstrated repeatedly (O'Hagan, 1997). Recent studies have shown that microparticles exert adjuvant effects for cell-mediated immunity, including the induction of cytotoxic-T-cell responses, which are desirable for the immunotherapy of cancer, after both systemic and mucosal administration (Maloy *et*

al, 1994; Moore *et al* 1995). At present it is not clear whether particulate systems will have advantages over other formulations for DNA vaccines but the prospect of delivering to phagocytic cells is exciting.

This chapter explores the possibility of using albumin microparticles produced by Quadrant's proprietary spray drying process, as carriers for DNA vaccines. Albumin is the most abundant protein circulating in blood, being an important carrier for drugs and endogenous substances like fatty acids, bilirubin and some hormones. There has been great interest shown in the ability of albumin microspheres to achieve specificity in drug delivery (Blake, 1974; Lohner, 1994). Albumin microparticles are a potentially useful means of delivering drugs to phagocytic cells because they will remain intact until they are taken up, but should ultimately be degradable, and are amenable to preparation in large batches (Blake, 1974; Lohner, 1994). As yet they have not been used to deliver DNA.

4.2. METHODS

Microparticles used in this work were prepared during a period of secondment at Quadrant (formerly Andaris), using established methods.

4.2.1. Preparation of cross-linked microparticles

The cross-linked microparticles were prepared in 3 steps, the first involved the removal of fatty acids from human serum albumin using activated charcoal, to create potential binding sites for cationic lipids which would in turn allow loading of DNA. Secondly concentration of the defatted human serum albumin (DHSA) was concentrated via diafiltration to reduce the volume of solution that was required to be spray dried. Finally the DHSA was spray dried to create microparticles which were subsequently subjected to heat fixation to create the cross-links within the microparticles. The process produced particles, which were solvated by water but remained intact in physiological buffer solutions.

4.2.1.1. Defatting of Human Serum Albumin by activated Charcoal (Chen, 1967)

8000ml of 5 %w/w human serum albumin (Red Cross), was concentrated via diafiltration (Amicon) to 4000 ml (2000 ml of HSA was passed through the diafiltration unit at a time). This solution was split into 2 x2000ml and each batch was washed with 10 L of purified water first, then re-concentrated and pooled resulting in a final volume of 2000 ml at approximately 20 % w/w HSA. The HSA was then defatted. For defatting the 2000 ml was divided into 2 and 125 g of activated charcoal (Sigma) was added to each batch. The pH was reduced to 3.0 using concentrated HCl. The mixture

was placed in an ice bath, stirring continuously for 1 hour, and then centrifuged for 20 minutes at 10000rpm. The supernatant was then filtered through a Whatman no. 54 filter paper on a Buchner funnel under slight vacuum. This was repeated until the solution was clear. The final protein concentration was established via the Biuret test. Finally the solution was filter sterilised into sterile containers ready for spray drying.

4.2.1.2. Biuret Assay

A protein standard of 5 mg albumin/ml was freshly prepared. The Biuret reagent consisted of 3 g copper sulphate and 9 g sodium potassium tartrate in 500ml of 0.2 mol/litre sodium hydroxide to which was added 5 g potassium iodide and made upto 1 litre with 0.2 mol/litre sodium hydroxide. The reagent was placed in a water bath at 37 ° C for 20 minutes. For assay 3ml of the biuret reagent was mixed with 2 ml of the protein solution and incubated at 37 ° C for 10 minutes. The solution was then cooled and the absorbance was determined and compared with the standard solution at 540 nm. A standard curve can be found in appendix *C4*.

4.2.1.3. Spray Drying of Defatted Human Serum Albumin

Spray drying was achieved by adapting a low temperature spray drying process developed by Quadrant Healthcare plc (formerly Andaris Ltd) at Quadrant Healthcare plc, Nottingham, UK which was similar to that described in Chapter 2. To produce albumin microparticles the Niro Mobile Minor spray dryer was used as larger volumes were being spray dried. The spray dried microparticles in the collection vessel were then transferred to an oven and held at 175°C for 55 minutes for heat fixation. The

microparticles were then blended with Mannitol (Sigma) at ratio of 4 part mannitol to 1 part microparticles to increase bulk density.

4.2.2. Physical Characterisation

4.2.2.1. Scanning Electron Microscopy (SEM)

The DHSA microparticles were examined for particle shape and surface characteristics. A representative sample of powder was mounted on aluminium specimen stubs using carbon-coated adhesive fixers. A thin, conductive layer of gold was evaporated onto the sample surface using a sputter coater (Model: S150B, Edwards High Vacuum, Crawley, UK) for 5 minutes. The prepared specimens were examined using the JEOL 6310 SEM (Japanese Electron Optics Ltd, Tokyo). Typical instrumentation settings, operating voltage 5-10 kV, spot size 12.

4.2.2.2. Particle Size Analysis

Particle sizing was performed using the MSX1 small volume unit Malvern Mastersizer X (Malvern Instruments Ltd., Malvern, UK, which had a maximum capacity of 20 ml. The particles were dispersed in acetone and sonicated for one minute. Then a representative sample was introduced into the sizing cell containing cyclohexane as the dispersant, drop wise using a pastuer pipette. The Mastersizer works on the principle of Franhofer Diffraction Theory as explained in Chapter 2. The laser passed through a lens with focal length of 100 mm (measurement range 0.2-180 μm). Particle size was calculated by volume.

4.2.3. DNA Loading onto DHSA Microparticles

In order to load DNA onto the microparticles, it was necessary to introduce a cationic functional group onto the surface of the microparticles to change the charge on the surface so that DNA could be adsorbed. The initial approach was to bind cationic lipids into the fatty acid binding site of the HSA.

4.2.3.1. Lipids

4.2.3.1.1. DOTAP

N-[-1-(2,3-Dioleolyoxy)propyl]-N,N,N-trimethylammonium-methylsulphate (Avanti Polar Lipids, USA). DOTAP powder was used to prepare liposomes as described in Chapter 2.

4.2.3.1.2. Alkyl trimethylammonium bromides

Dodecyltrimethylammonium bromide C15H34NBr (C_{12}TAB), tetradecyltrimethylammonium bromide C17H38NBr (C_{14}TAB), hexadecyltrimethylammonium bromide also known as cetrimide USP C19H42NBr (C_{16}TAB), were all purchased from Sigma, UK. The longer the alkyl-chain the higher the binding affinity.

4.2.3.2. Plasmid DNA

Plasmid DNA pCMV*luc* was propagated, purified and isolated as described in Chapter 2.

4.2.3.3. Collection of Microparticles from Blend

50 mg of powder blend was re-suspended in 1ml of purified water, allowing the sample to resuspend on a roller mixer for 5 minutes. The samples were centrifuged for 3 minutes at 3000rpm, and the supernatant was then removed and the pellet was re-suspended again in purified water and washed until three washings had been performed.

4.2.3.4. Loading of Lipid

The required quantity of lipid was dissolved in 1 ml of purified water. (If the lipid was insoluble in water, it was dissolved in chloroform, dried and reconstituted in water). The defatted HSA microparticles were washed and re-suspended with the lipid suspension and placed on a roller mixer for 30 minutes. Subsequently the samples were centrifuged for ten minutes at 3000rpm. The supernatant was removed and the pellet re-suspended in a further 1 ml of purified water. The suspension was centrifuge for 10 minutes at 3000 rpm and again the supernatant, was discarded. The pellet was air dried before further treatment.

4.2.3.5. Monitoring DNA loading via Nucleic Acid Staining

As DNA and albumin both absorb at 280 nm it was difficult to monitor the loading of DNA onto albumin by a direct method. The assay of DNA at low concentrations in the presence of the particles, was compromised by the leaching of small amounts of protein from the particles. The binding of DNA could be estimated, if the wavelength at which the DNA 'absorbed' could be shifted further up the UV spectrum. This was achieved by labelling plasmid DNA with a nucleic acid stain, YOYO-1, (Molecular Probes) which has a strong binding affinity for DNA and can provide ultra-high detection sensitivity for nucleic acids (Molecular Probes, UK). In addition to its high-affinity binding it is essentially non-fluorescent in the absence of nucleic acids and exhibits a large increase in fluorescence upon DNA binding.

The fatty acid content, of different commercial preparations of HSA are known to vary (Birkett *et al*, 1978). Also it is difficult to establish the number of fatty acids binding sites on a single albumin molecule as fatty acids are not permanently bound to albumin molecules but in equilibrium. It was estimated that HSA contained one fatty acid binding site per molecule of HSA, on this assumption cationic lipid DOTAP with on positive charge would bind to the fatty acid binding site, and cause a charge reversal from negative to positive. The latter in turn would create a charge environment to adsorb the negatively charged DNA (one negative charge) to the positive charges provided by the cationic lipid. Since these particles were to be assessed *in vitro* eventually a charge ratio of 2.5 for DNA/DOTAP was adopted (see Chapter 2). The microparticles were prepared as mentioned above loading DOTAP calculated to a charge ratio of 2.5 on the assumption that 10 μg of DNA would be loaded onto 10 mg of DOTAP loaded microparticles. The ratio of plasmid to YOYO-1 was chosen to be 50

base pairs:1 dye ratio as this was the most concentrated ratio that could be detected via spectrophotometry without leaving the linear range. Microparticles were incubated for 2 hours in a solution of plasmid/dye at concentration of 50 : 1 base pair/dye ratio respectively. The microparticles were then collected by centrifugation and absorbance readings of the supernatants were taken at excitation 491nm and emission 509nm. The control sample consisted of the original concentration of DNA/YOYO-1 without the presence of microparticles. Standard curve can be found in appendix *D*. Quantity of plasmid bound to the microparticles was calculated using the following equations: -

Equation 1:

Control sample absorbance – Test sample absorbance = Absorbance due to bound DNA

Equation 2:

$$\frac{\text{Absorbance due to bound DNA}}{\text{Absorbance of Control Sample}} \times \text{Concentration of DNA in sample} = \text{Concentration of DNA bound}$$

4.2.3.6. Polycations which were grafted onto DHSA Microparticles

4.2.3.6.1. Poly-L-lysine hydrobromide

The synthetic cationic polypeptide (average molecular weight of 30000 – 70000 Da) purchased from Sigma, UK.

4.2.3.6.2. Protamine

The natural cationic polypeptide protamine (calculated molecular weight 4100) was purchased from Sigma, UK, as an extract from salmon.

4.2.3.7. Grafting of Polycations onto the DHSA Microparticles

50 mg of the DHSA microparticles were incubated with either 0.1, 1, or 10 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) (Sigma, UK) and 5 mg of a cationic polymer dissolved in 5 ml of Milli-Q water for 30 minutes on a shaking incubator. EDAC is a water-soluble carbodiimide crosslinking reagent, which couples carboxylic acid groups to amines, forming an amide linkage.

4.2.3.8. Measuring the Zeta Potential

The zeta potentials of poly-L-lysine-DHSA microparticle complexes and protamine-DHSA microparticles produced using different cross linking reactions were determined using an electrophoretic method using the Malvern Zetasizer 3 with Multi 8 computing correlator (Malvern Instruments, Malvern UK). Measurements were possible in water at a concentration of 250 μg of particles per ml. All glass and plasticware was washed three times with pre-filtered water to reduce particulate contamination. For each batch of polymer grafted microparticles, mean particle electrophoretic mobility was measured in a thermostatically controlled micro-electrophoresis cell (AZ4) equilibrated to 25 °C. Three measurements were made for each batch. The instrument uses the Smoluchowski equation to calculate the zeta potential.

4.2.3.9. DNA Loading onto Polycation Coated DHSA Microparticles

Poly-L-lysine or protamine coated microparticles were incubated with pCMV*luc*. 1.5 mg of each batch of microparticles were incubated in a solution of 12, 24, 48, and 96 μg labelled DNA in 1.5 ml of PBS (at a DNA/YOYO-1 ratio of 50:1 as described earlier). Microparticles were incubated with the DNA solution for 2 hours on a shaking incubator, and subsequently collected by centrifugation at 3000 rpm. The supernatant was analysed for the DNA remaining in the solution, thus enabling the calculation of the quantity of DNA loaded onto the microparticles.

4.2.3.10. In Vitro Transfection of RIF-1 Cells

RIF-1 cells were cultured to carry out *in vitro* transfection as described in Chapter 2. 1.5 mg of microparticles grafted with poly-L-lysine at polymer microparticle ratio of 50:1, were incubated with pCMV*luc* at 7 μg/1.5 ml and 21 μg/1.5 ml in PBS for 2 hours on a shaking incubator. After 2 hours the microparticles were collected via centrifugation at 3000 rpm. The DNA loaded microparticles were redistributed in 500 μl of HBS. For transfection experiments, the negative controls were naked pCMV*luc* at 7 μg and 21 μg in 500 μl of HBS, and the positive controls were, pCMV*luc*/DOTAP complexes at charge ratio of 2.5 for both 7 μg and 21 μg of plasmid in the same volume (500μl). The transfection complexes were incubated with the RIF-1 cells under OptiMEM for 4 hours. The transfection medium was then removed and replaced with complete medium for a further 24 hours. After 24 hours the RIF-1 cells were harvested for analysis of luciferase activity and protein content.

4.3. RESULTS

4.3.1. Spray Drying of Defatted Albumin

Spray drying of DHSA or 2% protamine/DHSA particles was performed using the Niro Mobile Minor and adopting the 'open cycle' process. The conditions are documented in the tables below (Tables 4.1a-d). Batches of 59.0 g of DHSA and 10.1 g 2 % (w/w) Protamine/DHSA were spray-dried respectively. Tables 4.1a-d also show that the conditions during spray drying of both compounds were controlled rigorously. The outlet temperature, which would be expected to greatly influence the morphology of the final product, remained fairly constant throughout the spray drying cycle; at 97.5-98.0 °C for DHSA and 94.0 –93.7 °C for 2 % (w/w) Protamine/DHSA. The percentage recovery was quite low, which indicated that improvement in the efficiency of the spray drying process could have been made. The percentage of mass recovered for DHSA was 15 % and for 2%(w/w) Protamine/DHSA was 26.24%.

Tables 4.1. a-d Spray Drying Conditions

(a) Feed Material:

Conditions	Defatted –HSA	2% Protamine/Defatted –HSA (w/w)
Material Type	*Defatted –HSA*	*2% Protamine/Defatted –HSA(w/w)*
Total Protein Concentration (%w/v)	13.62	11.99
Volume (ml)	500	100
Mass Spray-dried (g)	59	10.1

(b) Feed Details

Conditions	Defatted –HSA	2% Protamine/Defatted –HSA (w/w)
Pump settings	30 rpm	30 rpm
Tube Detail (mm)	0.8D/1.6D	0.8D/1.6D
Feed Rate (g/min)	5.1	7.43

(c) Drying Conditions

		Defatted –HSA	2% Protamine/Defatted –HSA (w/w)
Inlet Temperature (°C)		220	220
Outlet Temperature (°C)	Start	97.5	94.0
	End	98.0	93.7
Atomisation Type		Twin-fluid	Twin-fluid
Atomisation Pressure (barg)		7.5	7.5

(d) Recovery

	Defatted –HSA	2% Protamine/Defatted –HSA (w/w)
Mass of Vessel (g)	594.1	594.1
Mass of Vessel + Product (g)	602.9	596.7
Mass Recovered (g)	8.9	2.7
Recovery (%)	15.0	26.2

4.3.2. Scanning Electron Microscopy

The surface morphology of the two batches of spray dried defatted albumin microparticles were examined using scanning electron microscopy. Typical images of DHSA microparticles and 2%(w/w) Protamine/DHSA microparticles are shown in Figure 4.1. The microparticles from both batches appear to have smooth surfaces with some particles containing internal voidages i.e. they are donut in shape. There appeared to be some aggregation within both batches, the DHSA alone being more aggregated than the 2% (w/w) Protamine/DHSA. The size ranges of the microparticles of both batches appeared to be between 1-10 μm. The crystalline fragments that can be observed in both batches is the mannitol they were blended with to increase bulk density.

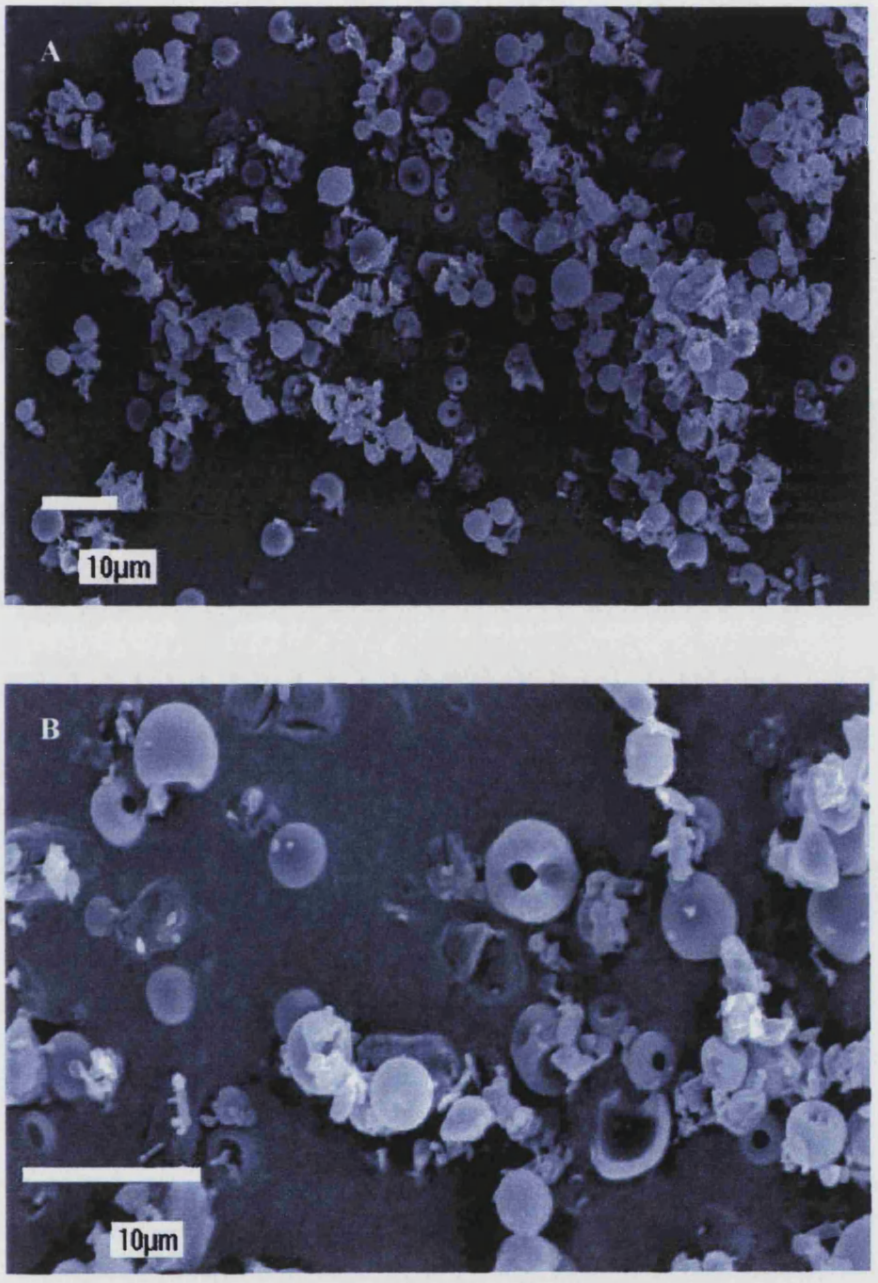


Figure 4.1. A and B are typical scanning electron microscopy images of spray dried defatted human serum albumin microparticles.

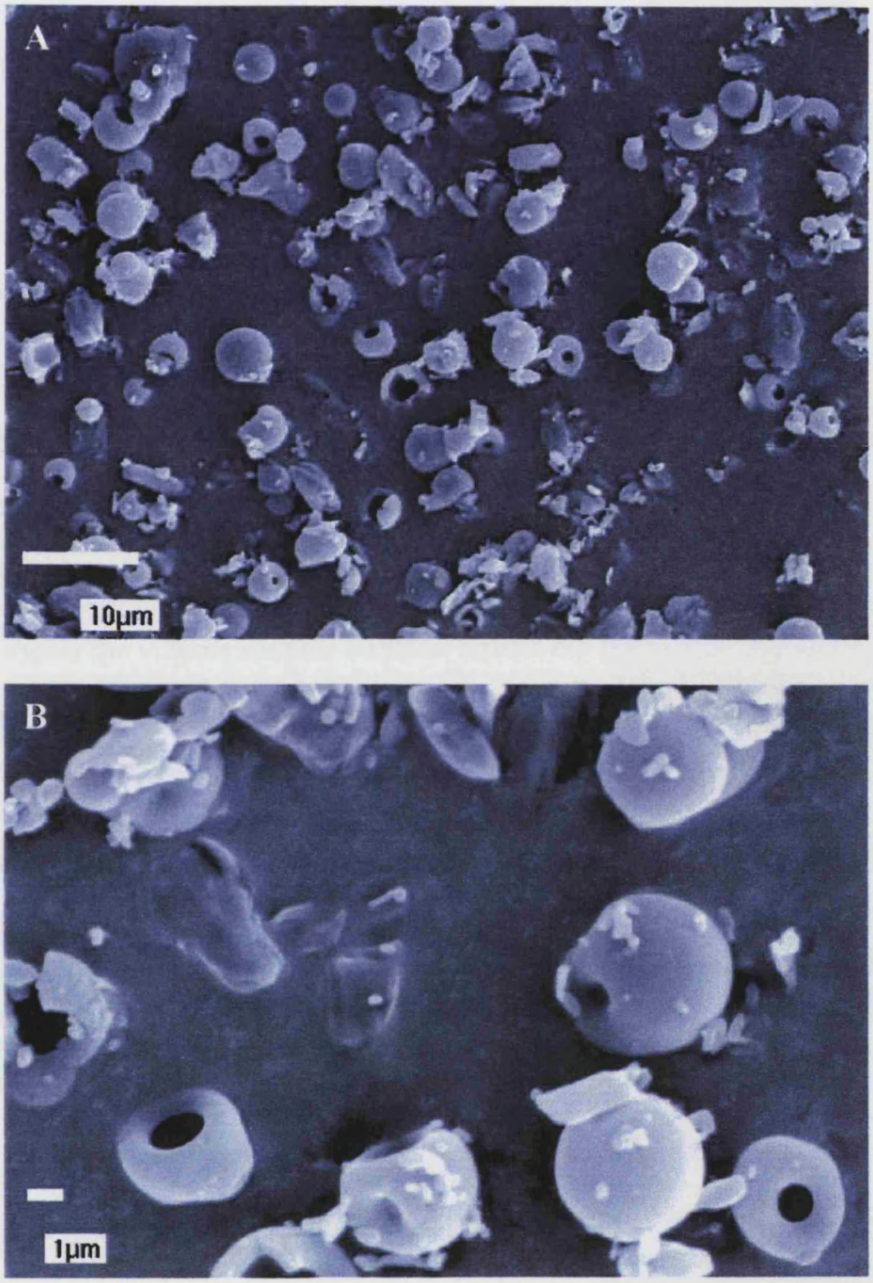


Figure 4.2. A and B are typical scanning electron microscopy images of spray dried defatted human serum albumin microparticles with 2%(w/w) Protamine.

4.3.3. Particle Size Analysis

Particle size analysis of the DHSA microparticles and the 2%(w/w) Protamine/DHSA microparticles was determined using the MSX1 small volume unit Malvern Mastersizer X. This system measures particle size according to the principle of the Fraunhofer Diffraction Theory, which was explained in Chapter 2. The particles were dispersed in cyclohexane and background readings were taken of the cyclohexane as a control, before actual measurements of the microparticles were taken. The data below shows the volume median diameter values for the samples analysed. The values at 10, 50, and 90% levels represent the percentage cumulative undersize for the samples tested. Therefore, the value quoted at 10 % indicates that 10 % of the particles are smaller than that value and 90 % are greater than that value in the volume analysed. Table 4.2 shows that before sonication of the samples the particles were aggregated and gave a very skewed distribution. However, after sonication the aggregated particles were dispersed to give a truer estimate of the particle size distribution.

Table 4.2. Particle Size of Spray Dried DHSA and 2%(w/w) Protamine/DHSA

		Mean Particle Size (μm)		
Sample ID	Sonication Time	D(0.1)	D(0.5)	D(0.9)
Defatted HSA	0 min	2.58 (±5.3)	6.19 (±11.1)	99.43 (±57.3)
	1 min	1.72 (±2.5)	3.53 (±1.0)	5.93 (±3.2)
2%Protamine/ DHSA (w/w)	0 min	2.91 (±9.1)	8.07 (±27.7)	126.21(±26.9)
	1 min	1.71 (±3.7)	3.66 (±1.9)	5.98 (±2.8)

(NB: all samples had 3 replicates, values in brackets are relative standard deviation)

4.3.4. Nucleic acid Staining

To monitor the loading of DNA onto the lipid loaded DHSA microparticles, it was necessary to label the DNA with a probe, which allowed the unbound DNA to be detected by spectrofluorimetry. One method, which has been developed over the past 5 years is to stain nucleic acids with a cyanine dye (Molecular Probes, UK). Cyanine dyes have unique properties, which make them an ideal choice for monitoring DNA loading. They are weakly fluorescent in the absence of nucleic acids but exhibit significant fluorescent enhancement upon DNA binding across the cyanine dye family (a possible increase from 100 to 1000 fold). This compares favourably with the fluorescence enhancement of thiazole orange upon DNA binding (~3000 fold). In this study the cyanine dye YOYO-1 was chosen which exhibits absorption/emission maxima of 491/509 when bound to double stranded DNA. YOYO-1 has ultra-high detection sensitivity (can detect YOYO bound DNA at concentrations as low as 100 pg/μl) (Rye *et al*, 1992) and produces stable complexes once bound. It has been found to exhibit at least two distinct binding mechanisms. At low dye:base pair ratios, the binding mode appears to consist primarily of intercalation. At high dye:base pair ratios, a second mode involving external binding begins to contribute (Haugland, 1996). To establish the time taken for the YOYO-1 to bind to plasmid DNA and to ensure the stability of complex once formed, samples of YOYO-1 and plasmid DNA were prepared at molar ratios of 50 base pair:1 molecule of YOYO-1 (Manufacturer's Protocol, Molecular Probes, UK) in sterile Milli-Q water. Absorbance readings were taken from time 0 – 220 minutes. In order to ensure minimal loss of dye through adsorption onto the surfaces of containers, samples were prepared in plastic containers with a minimum of transfer steps, as recommended by the manufacturer's protocol.

Figure 4.3 shows that the staining of the nucleic acid appears to have occurred almost immediately and remains very stable over the 3 hours and 40 minutes observed.

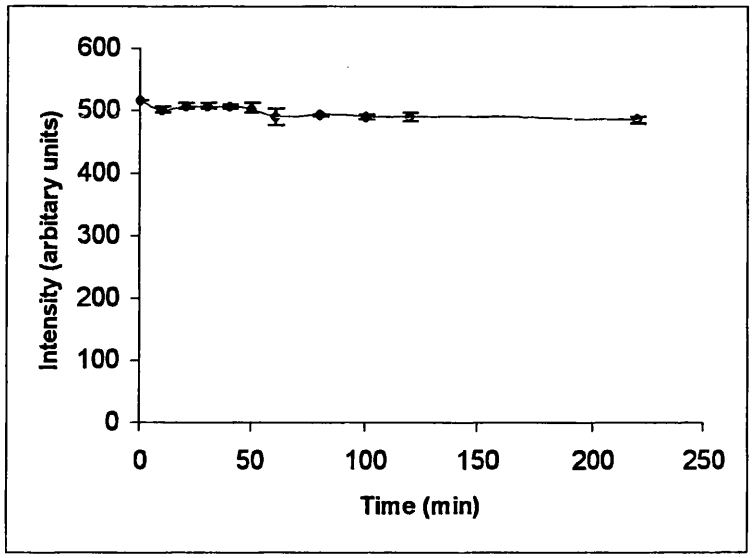


Figure 4.3. Time taken for staining of pCMV*luc* by YOYO-1 to reach equilibrium. A concentration of 50 base pairs : 1 dye ratio was monitored using a spectrofluorimeter at Em/Ex 491/509 over a period of 3 hours and 40 minutes. Data represents a mean of triplicate samples $\pm$SEM.

4.3.5. DNA Loading onto DHSA Microparticles

The loading of plasmid DNA was monitored using DNA/YOYO-1 complexes as described in section 4.3.4. 15 mg of DOTAP loaded DHSA microparticles were incubated for 2 hours with 1.5 ml DNA/YOYO-1 solutions at concentrations from 1-10 μg/ml. Absorbance readings were taken at excitation/emission maxima 491/509 and exact quantity of plasmid loaded onto the microparticles was calculated with the aid of the method described in section 4.2.3.5. Figure 4.4 shows a control experiment to show that DNA was removed by the lipid loaded microparticles. Figure 4.5 shows that the loading of DNA onto DOTAP loaded microparticles was generally less than 1.5 μg/15 mg. An adsorption isotherm was obtained with considerable scatter due to the weak interaction. The highest quantity loaded was in the region of 1.5 μg per 15 mg of microparticles too low to be practical. Doses of DNA in animal experiments are typically 20-100 μg DNA, which would require 300-1500 mg particles.

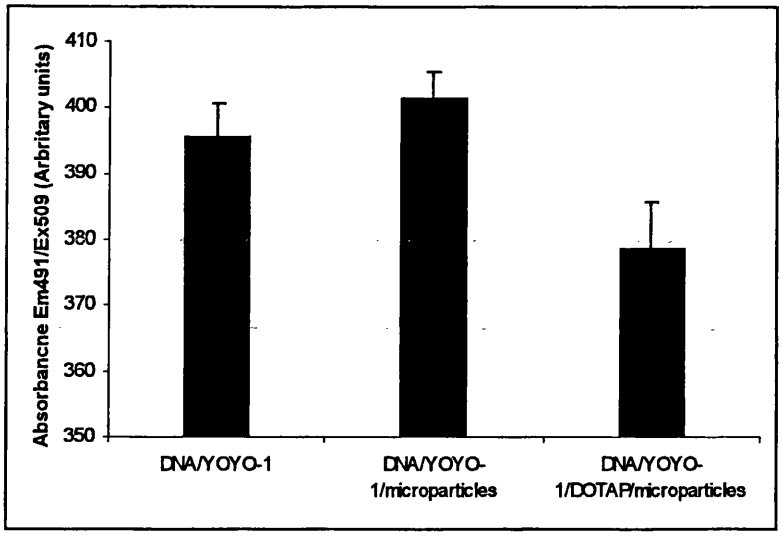


Figure 4.4. Control experiment to show that the cationic lipid DOTAP adsorbs DNA onto the surface of the microparticles. Absorbance readings were taken of samples incubated for 2 hours of DNA/YOYO-1, DNA/YOYO-1/microparticles, and DNA/YOYO-1/DOTAP/microparticles. Data represents the mean of triplicate samples ±SEM.

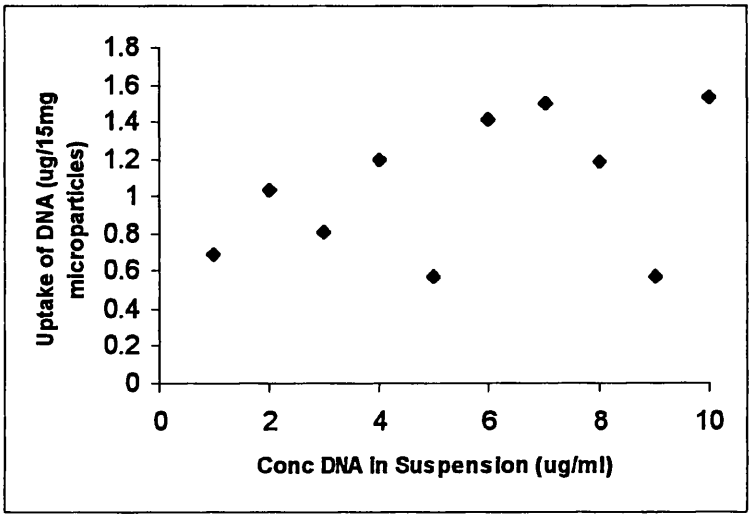


Figure 4.5. Plasmid loaded onto defatted albumin microparticles. 15 mg deffated albumin microparticles were incubated with a plasmid and YOYO-1 complex solution for 2 hours, at varying concentration of plasmid from 0-10 μg/ml.

4.3.6. DHSA Microparticles Complexed with Different Mono Alkyl Cationic Surfactants

As an alternative to DOTAP the DHSA microparticles were loaded with mono alkyl cationic surfactants to see if there could be an improvement in the amount of plasmid that could be loaded onto these microparticles. These mono alkyl surfactants were micellar and could be complexed from solution. The DOTAP loaded DHSA microparticles were used as a control. 15 mg samples of microparticles were loaded with 79.875 μg of DOTAP, C_{12}TAB, C_{14}TAB, or C_{16}TAB. Subsequently the lipid-loaded DHSA microparticles were incubated for 2 hours in a 1.5 ml DNA/YOYO-1 solution at a concentration of 10 μg/ml DNA. The quantity loaded onto DHSA microparticle was determined as described in the previous section. Figure 4.6 shows that the mono alkyl surfactants were more successful at loading plasmid DNA onto the surface of the microparticles than the dialkyl cationic lipid (DOTAP). The longer chain length of these CTAB lipids appeared to increase the binding of plasmid DNA, which reached 14.8 μg/15 mg with C_{16}TAB.

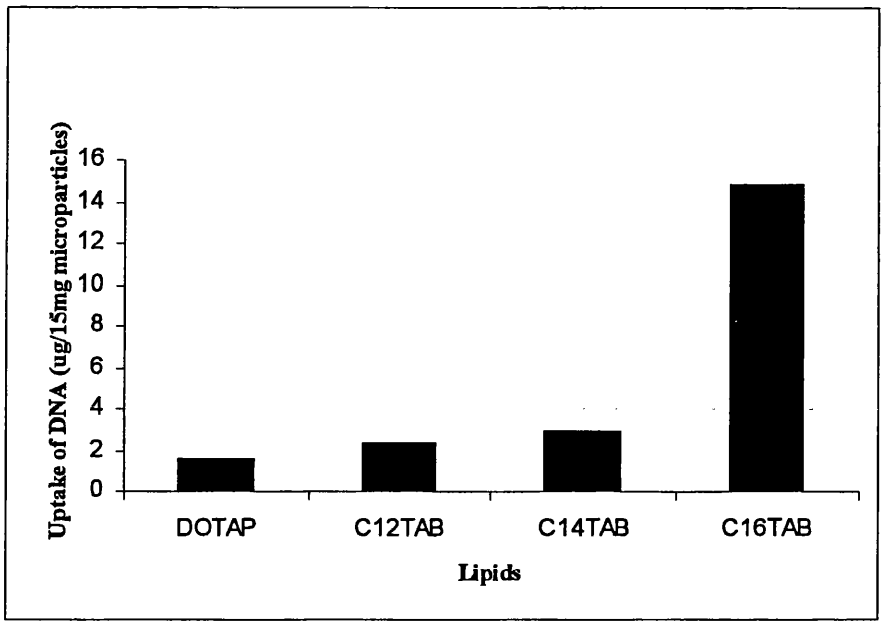


Figure 4.6. Mass of plasmid loaded onto defatted albumin microparticles complexed with different lipids. Defatted albumin microparticles were complexed with DOTAP, C_{12}TAB, C_{14}TAB, C_{16}TAB, and incubated for 2 hours in a plasmid/YOYO-1 solution at 10 μg/ml of plasmid.

4.3.7. Effect of DNA Loading by Varying Concentration of C_{16}TAB to DHSA Microparticles

In order to determine the optimum concentration of C_{16}TAB for particle complexation 15 mg samples of DHSA microparticles were loaded with varying concentrations of C_{16}TAB at 0.2, 0.5, 1.0, 2.0, 5.0, and 25.0 mg/15 mg DHSA microparticles in 1.5 ml. The C_{16}TAB loaded DHSA microparticles were then harvested and incubated for 2 hours, in 1.5 ml of a solution of plasmid DNA/YOYO-1 at a concentration of 10 μg/ml of DNA. The quantity of DNA loaded onto the C_{16}TAB-DHSA microparticles was determined as described previously.

Figure 4.7 shows that the concentration of C_{16}TAB used to load C_{16}TAB onto the surface of the DHSA microparticles had a marked influence on the quantity of DNA that can be loaded onto the surface of the DHSA microparticles. It appeared that C_{16}TAB loading was most efficient at 2 mg/15 mg microparticles which gave the highest mass taken up of 14.85 μg of plasmid DNA. Below 2 mg/15 mg microparticles C_{16}TAB was suboptimal. At C_{16}TAB concentration > 2 mg/15 mg microparticles the extent of DNA loading onto the surface of the microparticles appeared to decrease.

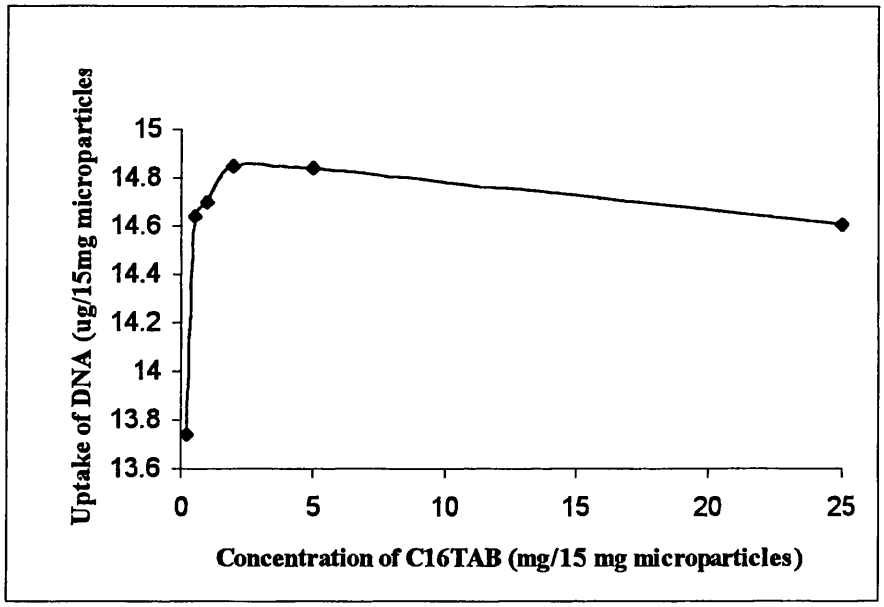


Figure 4.7. Plasmid loaded onto defatted albumin microparticles complexed with varying concentrations of C_{16}TAB. Defatted albumin microparticles were complexed with C_{16}TAB at 0.2, 0.5, 1.0, 2.0, 5.0, 25.0 mg/15 mg microparticles in 1.5 ml PBS and incubated for 2 hours in a solution of plasmid/YOYO-1 at 10 μg/ml concentration of plasmid.

4.3.8. Zeta Potential of Polycation Grafted Microparticles

Zeta potential is the potential at the surface of shear between the charged surface and electrolyte solution. Surfaces of particles dispersed in a solvent become charged as a result of the adsorption of ions or ionisation of surface groups. The effects generate an electrical double layer around the particle in which the potential is highest at the particle surface, reaching zero in bulk solution. The inner layer known as the Stern plane consists mainly of counter ions attracted to the surface by electrostatic or Van der Waals forces and solvent ions. The outer layer, the diffuse layer extends a distance from the particle, which is determined by the surface charge on the particle, and the nature and concentration of the ions in the surrounding electrolyte (Shaw, 1994).

Some ions are more or less rigidly attached to the particle and when exposed to an electric field each particle will move toward the appropriate pole carrying with it those ions sufficiently attracted to the surface. The boundary between the moving particles with its bound counter-ions and the surrounding solution constitutes the shear plane. It is virtually impossible to measure the potential at the particle surface or at its stern layer. However, one can measure relatively easily the potential at the shear plane. The potential measured at this plane is termed the zeta potential.

The surface charge of the microparticles is expected to influence its interactions with various biological and chemical components, as well as its distribution, access and entry into target cells. The zeta potential of the defatted human serum albumin microparticles without and with grafted polymers poly-L-lysine and protamine were investigated (see Table 4.3).

Table 4.3. Mean zeta potentials of DHSA microparticles and those coated with polycations. 3 replicate measurements were taken.

Sample		Mean Zeta Potential (mV)	Standard Deviation
DHSA Microparticles (uncoated)		-53.0	1.0
2% Protamine/DHSA Microparticles		-40.6	1.2
Microparticle/Poly-L-lysine/EDAC (MpLLE) mass ratio (mg)	50:5:0.1	+35.8	0.9
	50:5:1.0	+32.9	1.9
	50:5:10.0	+39.5	0.5
Microparticle/Protamine/ EDAC mass ratio (MPE) (mg)	50:5:0.1	+20.3	1.0
	50:5:1.0	+14.6	1.5
	50:5:10.0	+18.8	2.2

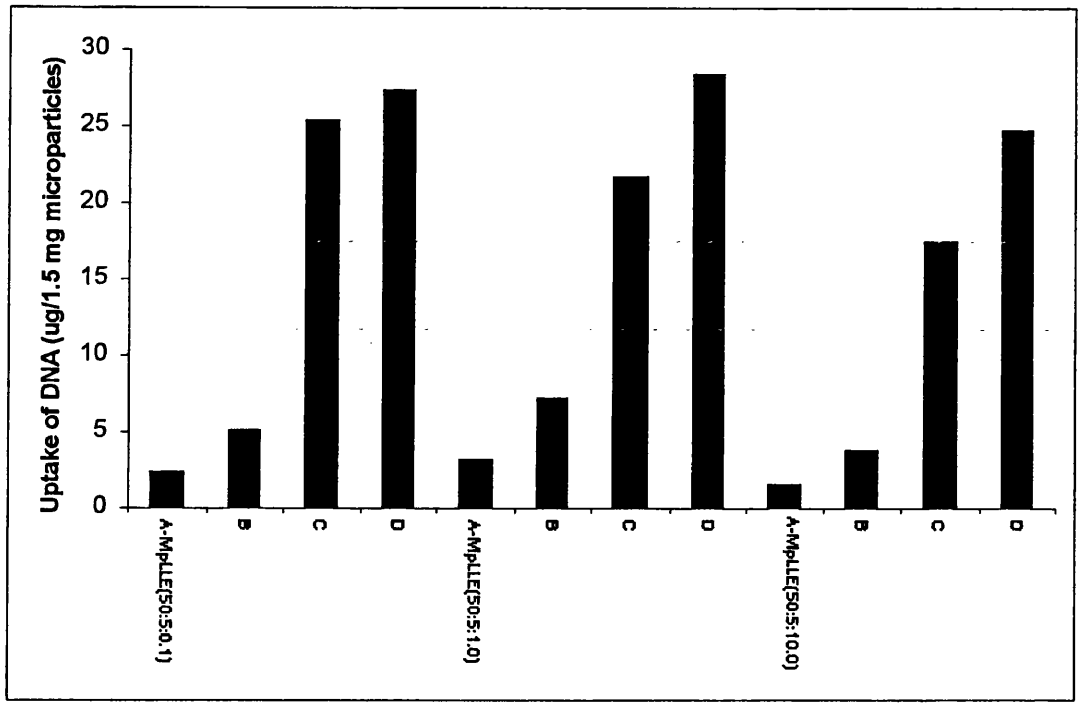
The DHSA microparticles exhibited a relatively high negative charge, and as expected, DHSA microparticles containing 2%(w/w) protamine, a cationic polymer, reduced the negative charge, though not enough to render the charge positive. Complexation of poly-L-lysine to the surface of the DHSA microparticles led to a clear change in charge on the surface to highly positive. The positive surface charge for poly-L-lysine complexed DHSA microparticles was similar regardless of the three conditions used for grafting.

The DHSA microparticles with protamine complexed to their surface also had a positive surface charge, and again regardless of the reaction conditions, the surface charge remained similar. However, the surface charge of DHSA microparticles complexed with protamine exhibited a lower positive surface charge than the DHSA microparticles complexed with poly-L-lysine.

4.3.9. DNA Loading onto Polycation Coated Microparticles

1.5 mg of either poly-L-lysine or protamine-coated DHSA microparticles produced using each of the 3 protocols were incubated with 12, 24, 48, and 96 μg of pCMV*luc* in 1.5 ml PBS for 2 hours (using DNA:YOYO-1 molar ratios of 50:1). The microparticles were collected via centrifugation and the supernatant was analysed for plasmid remaining, which allowed estimation of the quantity of plasmid loaded onto the microparticles (see section 4.2.3.5). The loading of plasmid DNA was generally similar for the 3 different batches of poly-L-lysine coated DHSA microparticles (see Figure 4.8). This was also true for the 3 batches of protamine coated microparticles. The poly-L-lysine coated DHSA microparticles generally loaded more plasmid DNA onto their surface than their protamine-coated counterparts. For protamine coated microparticles prepared at MPE ratios of 50:5:0.1 (mg) and 50:5:1.0 (mg), it was difficult to obtain an accurate measurement when microparticles were incubated with 96 μg plasmid DNA/1.5 ml. This was due to the relative amount of DNA remaining in the supernatant, which caused lack of precision in the assay.

a)



b)

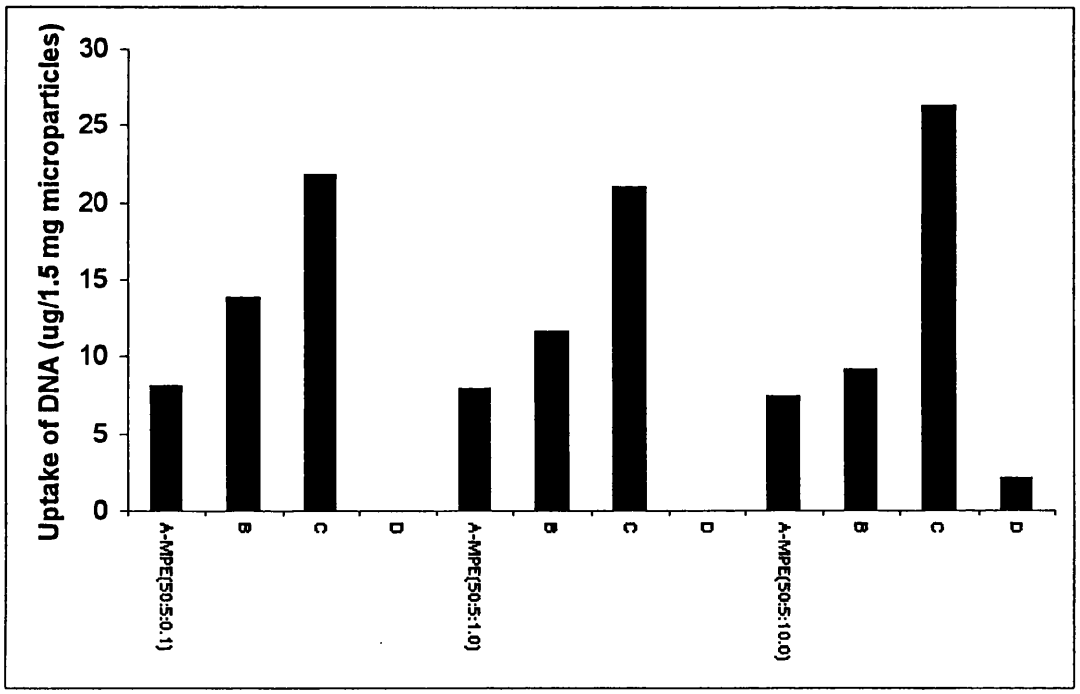


Figure 4.8.a &b Plasmid loaded onto defatted albumin microparticles complexed with poly-L-lysine (MpLLE) (w/w) (a) and protamine (MPE) (w/w) (b). (A) is incubation with 12 μg of pCMV*luc*/1.5 ml. (B) is incubation with 24 μg of pCMV*luc*/1.5 ml. (C) is incubation with 48 μg pCMV*luc*/1.5 ml. (D) is incubation with 96 μg pCMV*luc*.

4.3.10. *In Vitro* Transfection of RIF-1 cells with DNA Loaded Microparticles

The ability of the DNA loaded microparticles to transfect tumour cells was assessed by *in vitro* transfection of RIF-1 cells. 1.5 mg DHSA microparticles grafted with poly-L-lysine (MpLLE mass ratio 50:5:1) were loaded with 7 or 21 μg of pCMV*luc* in 1.5 ml of PBS as described earlier. The transfection complexes were incubated with the RIF-1 cells for 4 hours, the cells were then harvested 24 hours post transfection for analysis of luciferase activity and protein content.

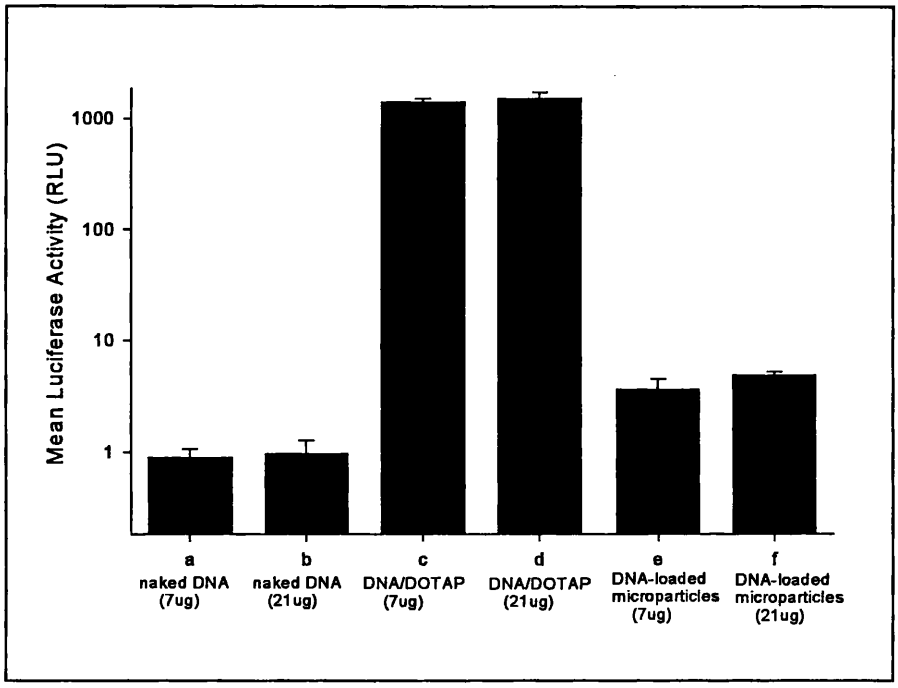


Figure 4.9. A logarithmic plot of luciferase expression after transfection with pCMV*luc* loaded microparticles. RIF-1 cells were incubated with the transfection complexes for 4 hours. The cells were then harvested 24 hours post transfection for analysis of luciferase activity. Data represents the mean of triplicate samples ±SEM.

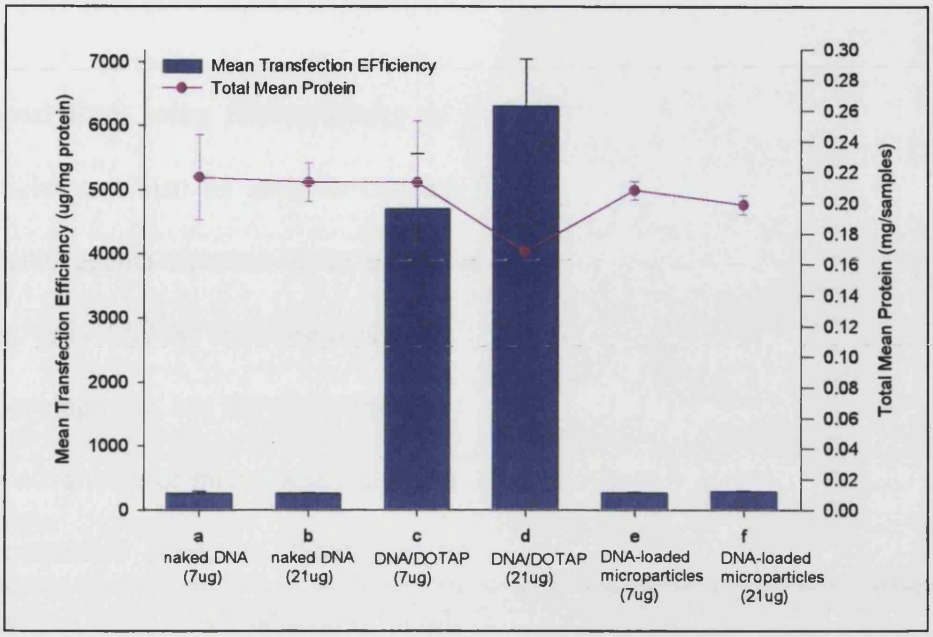


Figure 4.10. Luciferase expression after transfection of RIF-1 cells with pCMV*luc* loaded microparticles and the total mean protein produced per sample. The data represents the mean of triplicate samples ±SEM.

Figure 4.9 shows that some gene expression was obtained from the DNA-loaded microparticles.

4.4. DISCUSSION

Apart from using microparticles as carriers of therapeutic gene to the lung, microparticles can also be used as carriers for particulate DNA vaccines, acting as immunogenic agents themselves or acting as carriers for DNA delivery to antigen presenting cells (APCs) and macrophages. There appears to be great potential in vaccine development, but the mechanisms of action need to be studied in more detail. One clear advantage of microparticles is that they can be produced from biodegradable and biocompatible polymers. Controlled-release might enable the development of single-dose vaccine, and adjuvants might be entrapped simultaneously in the microparticles. Mucosal administration of microparticles can induce serum and secretary antibodies as well as inducing cell-mediated immunity.

This chapter has investigated the use of defatted human serum albumin microparticles as possible carriers for DNA vaccines. After defatting the HSA it was spray dried to produce microparticles which were then heat fixed by incubating the microparticles at 175°C. Heat fixation is known to cause cross-linking of the polymer matrices rendering the microparticles in soluble in aqueous medium (Arshady, 1990). The initial idea to removing the fatty acid from the human serum albumin was to create acyl chain binding sites, which would enable the loading of cationic lipids on the surface. Removal of the fatty acids was performed by the activated charcoal method described in section 4.2.1.1. The activated charcoal, possess a large surface area which enables the adsorption of the fatty acids from the albumin over time. This method was labour intensive and time consuming as well as being potentially quite harmful due to exposure low pH. An alternative would be the Lipidex 1000 chromatography method where fatty acids are removed by chromatography at 37°C on a column of Lipidex

1000. The latter method is milder and more rapid, since the harmful low pH and centrifugation step are not necessary; it has been reported to be more efficient (Glatz and Veerkamp, 1983).

Two batches of the defatted human serum albumin were spray dried, one batch contained 2%(w/w) protamine, a cationic polypeptide which is used in food products. Protamine was included to investigate whether the surface properties of the microparticles could be changed towards promoting an increase in DNA loading. The spray drying process used in both cases was Quadrants proprietary spray drying process, an 'open cycle' process using the Niro mobile minor, spray dryer and pneumatic atomisation (twin-fluid). The conditions of the spray drying process were well-controlled. The spray drying temperatures, the inlet temperature was fixed at 250°C, and the outlet temperature ranged from 93.7-98°C. The percentage recovery of the product was quite low for both batches, 15% for DHSA batch and 26.24% for the 2%(w/w) protamine/DHSA batch. The latter higher yield achieved in this case could be due to the fact the polymer in the second batch was more concentrated due to the smaller volume. The low product recovery could be due to several possibilities, as loss generally occurs after the spray-dried powder is formed, due to process of separation of the powder from the circulating gas, in this case it was the cyclone method. The main drying chamber had a very large diameter and hence large surface area, and it has been reported that a significant build up of powder can occur on the internal walls of a spray drying unit. This problem can be particularly prevalent with the fine particles of drugs, which exhibit adhesion forces to the wall that are much greater than the shear stresses imposed by the velocity field of the gas. Although tapping the main chamber and joint areas of the pipe-work, which is known to help, there will always be a thin layer that

cannot be dislodged by this method (Saatchi and Van Oorte, 1996). The latter problem could also be overcome by increasing gas inlet velocity and increasing mass loading.

The microparticles were visualised using SEM to investigate morphology and surface characteristics (see Figures 4.1 and 4.2). From the SEM images it can be observed that both batches of the DHSA without and with protamine produced spherical hollow 'doughnut' like microparticles under the spray drying conditions used. As mentioned in Chapter 2 this phenomenon is governed partly by the conditions used during the spray drying process in particular the relationship between inlet temperature and the boiling point of the solvent, and is partly the result of the chemical nature of the solute. The doughnut shape, usually comes about through rapid drying in the initial stages, which would have come about as a result of the relatively high inlet temperature (250°C) which was substantially higher than the boiling point of droplet solution in this case water (100°C), and later due to the chemical nature of the human serum albumin (Sacchetti and Van Oorte, 1996, Maa *et al*, 1997).

For use of microparticles as vaccine adjuvants/carriers of DNA vaccines, the particle size needs to remain in the size range of <10 μm as it has been reported that smaller microparticles were significantly more immunogenic than larger particles (O'Hagan, 1993). Table 4.1 shows the volume median diameter of the microparticles produced by both batches. Generally, the particle size distribution at 50% cumulative under size fell in the range of 3.53-3.66 for both batches. This is well within the range required for these microparticles to be suitable as carriers for vaccines.

In order to monitor loading of plasmid DNA onto the surface of the DHSA microparticles, a method had to be developed. As DNA and albumin essentially both absorb at 280 nm it was difficult to monitor the loading of DNA onto albumin directly by spectrophotometry (Plummer, 1987). One way around this problem was to

artificially create a chromophore for the plasmid by intercalation of a higher-affinity fluorophore. In 1990, Glazer and Rye reported that complexes of ethidium homodimer (EthD) with double-stranded (dsDNA), allowed fluorescence and quantitation of DNA fragments on agarose gels (Glazer and Rye, 1992). These observations and other reports of stability of complexes between DNA and other appropriately linked dimers or oligomers of intercalating-compounds, suggested that this phenomenon could be exploited to generate a family of highly fluorescent stable dsDNA-dye complexes with distinctive properties, which could have many analytical applications. This led to attention to asymmetric cyanine dyes, thiazole orange (TO) and oxazole yellow (YO) an analogue of thiazole orange. When free these dyes are weakly fluorescent but upon binding to DNA can give rise to a 3,000 fold enhancement of fluorescence, and binding is readily reversible (Rye *et al*, 1992). Further investigation led to suggestions that unsymmetrical cyanine dyes such as thiazole orange or its analogues could be bridged through bis-cationic linker similar to that linking the chromophores in ethidium homodimer to produce dimeric dyes, which led to the development of a thiazole orange dimer (TOTO) and an oxazole yellow dimer (YOYO). YOYO-1, commercially available from Molecular Probes, was chosen as a suitable intercalating dye as it is essentially non-fluorescent in the absence of DNA, gives rise to a 1000 fold increase in fluorescence upon binding to DNA and is very stable (Molecular Probes). Complexation appeared to occur almost immediately and remained stable for at least 3 hours and 40 minutes, the length of time monitored.

In order to load any DNA onto the surface of the DHSA microparticles, it would be necessary to coat the surface with a positive charge, because as expected removal of the fatty acids left the microparticles with negative surface charge, evidence of this is provided by the zeta potential data in table 4.2. Initial DNA loading experiments were

carried out by loading the acyl chain binding sites within the DHSA microparticles with a cationic lipid i.e. DOTAP which is known to interact with plasmid DNA (Felgner *et al*, 1994). Preliminary studies carried out for Quadrant indicated that this was a feasible approach to DNA binding. The results obtained here showed very little DNA bound to DOTAP-loaded microparticles, the highest DNA adsorption, lay in the region of 1.5 μg DNA/15 mg microparticles. The latter was probably due to the limited number of fatty acid binding sites being present for DOTAP to bind to. An alternative cationic lipid with non-specific binding was investigated, the alkyl trimethyl ammonium bromides (TABs) which will also bind such that the longer the alkyl chain the higher the binding affinity, which gives more flexibility on the quantity of plasmid to be loaded. C_{12}TAB, C_{14}TAB, and C_{16}TAB were investigated. The C_{16}TAB which had the longest alkyl chain of the three TABs investigated had the highest binding affinity, loading DNA in the range of 15μg/15 mg of microparticles. Still, relatively speaking the quantity of the DNA being loaded onto the surface of these microparticles was relatively low, and in order to provide therapeutic levels of DNA, it was thought that the microparticles must be able to load more than 15 μg/15 mg. Further experiments were carried out to determine whether increasing the quantity of cationic lipid loaded onto the surface of the microparticles results in an increase in the quantity of DNA loading onto the surface of the microparticles. It was accepted that in such circumstances not all of the CTAB would have been specifically bound. Results (see Fig 4.5) showed that increasing the exposure to C_{16}TAB from 0.2-2.0 mg/15 mg of microparticles lead to an increase in loading of the DNA. However, at concentrations of 5 and 25 mg/15 mg of microparticles no further loading of the DNA took place. This probably indicates that a saturation of the surface of the microparticles with the lipid may have occurred at exposure of 2.0 mg of C_{16}TAB to 15 mg of microparticles. It can be assumed that the

charge on the surface of these is more than likely to be similar, regardless of the quantity of cationic lipid loaded, another reason for there not being an increase in the loading of DNA.

As it appeared that cationic lipids may not be the choice of adjuvant to promote loading of DNA onto the surface of the DHSA microparticles alternatives were investigated. One approach was to spray dry a positively charged polypeptide along with DHSA. Protamine a polypeptide used in the formulation of potential non-viral gene delivery (Li *et al*, 1998) was spray dried with the DHSA to enable modification of the surface charge of the microparticles during microparticle formation. A batch of 2%w/w protamine/DHSA microparticles was produced, however it was found that the 2%w/w content of protamine was not a sufficient concentration to change the surface charge from negative to positive (see Table 4.3). It was clear that further batches of protamine/DHSA microparticles had to be produced with higher protamine content to investigate surface modification during microparticle formation, but this was not possible during the under taking of this research due to lack of access to equipment, time and money. Another alternative investigated was the grafting of cationic polymers onto the surface of the microparticles. Poly-L-lysine a polypeptide widely used in research towards developing a successful non-viral gene delivery vector (Wagner *et al*, 1990, 1991a) and protamine, were investigated for loading plasmid DNA onto the surface of the DHSA microparticles. The polymers were grafted onto the surface of the microparticles using a carbodimide strategy at ratios of 50 mg of microparticles to 5 mg polycation to 0.1, 1.0, and 10.0 mg of EDAC. EDAC is a cross-linking agent whose mechanism of action is based on the activation of the carboxyl groups, which then permits their cross-linking to amino groups (Jorge-Herrero *et al*, 1999). EDAC was used to facilitate the grafting of the polymers onto the albumin microparticles. The zeta

potentials of the polymer coated microparticles were measured. As expected, before coating with the polymer the DHSA microparticles had a negative surface charge. The polycation-coated microparticles all displayed positive surface charges, and raising the quantity of polymer available for the grafting reaction did not effect the final surface charges significantly. Poly-L-lysine coated microparticles had higher positive surface charges than those coated with protamine, which is possibly related to the chain length of the poly-L-lysine used compared to protamine.

The quantity of plasmid that could be loaded onto the surface of these polymer coated microparticles was investigated. Samples of polymer coated microparticles (1.5 mg) were incubated with 12, 24, 48 and 96 μg of pCMV*luc*/1.5 ml (1.5 ml was the minimum volume required to obtain a reasonable reading in the spectrofluorimeter). The poly-L-lysine coated microparticles bound more DNA onto the surface of the microparticles than those coated with protamine. In agreement with the zeta potential results increasing the ratio of polymer to microparticles did not appear to make a significant difference.

7 or 21 μg pCMV*luc* was incubated with poly-L-lysine coated microparticles produced using a reaction ratio (MpLLE) of 50:5:1 (w/w). These microparticles were then used for *in vitro* transfection of RIF-1 cells. Negative controls were 7 and 21 μg of naked pCMV*luc*, which is known to give little or no transfection. The positive controls were lipoplexes of pCMV*luc* /DOTAP using 7 or 21 μg of pCMV*luc* condensed with DOTAP at a charge ratio of 2.5. DOTAP is known to aid transfection, through condensation of plasmid DNA modifying its size, charge and surface characteristics, therefore enhancing the interaction of pCMV*luc* with the surface of the target cells; inducing endocytosis thus eventually leading to transfection. No transfection was obtained from naked DNA as expected, the pCMV*luc*/DOTAP lipoplexes gave rise to

gene expression between 4000-7000 μg/mg of protein. However, only 10-35 μg/mg protein of gene expression was obtained from the pCMV*luc* loaded microparticles. This was possibly due to poor uptake of large particles. Unless the polyplexes of poly-L-lysine/pCMV*luc* were shed by the microparticles, which may be the reason why some gene expression was obtained, there is very little chance of any transfection occurring. As the microparticles alone were probably too big to enter the RIF-1 cells which were cultured as a monolayer. Poly-L-lysine has been reported to give rise to transfection in a similar manner to cationic lipids (Wagner *et al*, 1991a) in various cell lines. However, it is also known to be toxic and to have very limited solubility 20 μg/ml (Wagner *et al*, 1998), which may also affect transfection efficiency. Protein levels were monitored through the transfection experiment (see Figure 4.10) and there appears to be relatively no change, ruling out possible effects through toxicity.

It is clear that DNA can be loaded onto these microparticles, but a lot more experiments need to be carried out to find the most suitable means of modifying the surface of the DHSA microparticles to obtain an optimal quantity of DNA that these microparticles can carry. The latter includes finding a suitable polypeptide, and either directly loading onto the DHSA microparticles or spray drying this polypeptide with the DHSA to produce microparticles.

CHAPTER 5

INVESTIGATION OF THE FATE OF ALBUMIN MICROPARTICLES AFTER INTRAMUSCULAR OR INTRATUMORAL INJECTION

5.1. INTRODUCTION

Particulate carrier systems such as albumin microparticles have the potential to deliver DNA vaccines (or indeed peptide antigens) to antigen presenting cells (APCs) or macrophages. This study is concerned exclusively with DNA delivery. Several gene expression studies have been carried out *in vitro* and *in vivo* to assess the ability of professional phagocytosing cells to phagocytose microparticles. Condon *et al* used the gene gun technology to deliver DNA-coated gold particles to the cutaneous dendritic cells (Langerhans cells) of mice. Some gold particles were subsequently formed located in the draining lymph nodes, where they were thought to be inside migrant cutaneous dendritic cells (Condon *et al.*, 1996). This suggests that a genuine immune response could be stimulated by such an approach. Other studies involved monitoring the behaviour of phagocytic particles such as neutrophil, monocytes and macrophages when incubated *in vitro* with microparticles (Wang *et al.*, 1999). Microparticle delivery studies have also involved the transfection of solid tumour models with microparticles loaded with reporter genes (Dass *et al.*, 2000).

This chapter explores the possibility of albumin microparticles inducing an immune response *in vivo* and the likelihood that these microparticles are taken up by APCs particularly macrophages. FITC labelled DHSA microparticles were assessed *in vivo* to investigate the relationship between the presence of the microparticles and the number of macrophages present in the tumour and muscle tissue. However, before *in*

vivo experiments were conducted it was considered sensible to carry out, *in vitro* experiments to determine whether macrophages were able to phagocytose the microparticles, and if so to determine how long macrophages take to phagocytose microparticles.

This chapter also describes investigations of the distribution of the microparticles in solid tissue after injection, and the length of time the particles reside in the tissue before degradation.

5.2. METHODS

5.2.1. FITC Labelling of DHSA Microparticles

In order to locate the microparticles *in vitro* and *in vivo* the microparticles were labelled with fluorescein isothiocyanate commonly known as FITC, which is a widely used reagent to attach a fluorescent label to proteins via the amine group.

100 mg of DHSA microparticles were suspended in 2 ml 1% Tween 80 solution, vortexed and left for 30 minutes to allow the microparticles to settle. Microparticles were collected via centrifugation for 2 minutes at 3500 rpm, then washed 3 times with 5 ml water and once with 5 ml 0.1M Tris buffer, to remove the detergent and excipients, collecting the microparticles after each was by centrifugation as before. To a suspension of DHSA microparticles in 1 ml 0.1M Tris buffer (pH 8) was added a solution of 35 mg fluorescein isothiocyanate (Sigma, UK) in 2 ml 0.1M sodium hydroxide solution (making sure the FITC had dissolved before it was added to the microparticles). The reaction mixture was stirred overnight at room temperature and the next day the microparticles were collected by centrifugation as before. The supernatant was removed and discarded, the microparticles were washed with 5 ml 0.1M Tris buffer until the supernatant was clear. Microparticles were collected each time by centrifugation as before. The microparticles were labelled with FITC and therefore appeared orange in colour.

5.2.1.1. Scanning Electron Microscopy (SEM)

SEM images were taken to observe particle shape and surface characteristics. Samples were prepared, and images were recorded under the same conditions as described in Chapter 4.

5.2.2. Isolation of Mouse Peritoneal Macrophages

The C3H mouse peritoneal cavity was injected with 2 ml of sterilised Hanks Balanced Salt Solution (37°C)(Mann, 1973). The abdomen was massaged for a few minutes. The peritoneal cavity was revealed and a small incision was made to withdraw the fluid in the cavity using a syringe without a needle. It was difficult to establish how many macrophages were present in the peritoneal cavity therefore in order to get a reasonable yield of macrophages several mice were needed, depending on the size of the experiment. Once all the samples were collected and collated the suspension was centrifuged at 1000 rpm for 5 minutes which was sufficient to sediment the cells without distorting them. The supernatant was poured away and the cells were resuspended in RPMI. A cell count was performed before plating out the cells in six well plates. Cells at $2x10^5$ were plated out and left to adhere overnight. Macrophages are reported to adhere to the surface of the plates therefore before experiments commenced the medium was replaced removing any cells that had not adhered to the surface. 1.5 mg FITC labelled DHSA microparticles, were introduced either in aggregated form or after sonication for 1 minute. Microparticles were incubated with the peritoneal macrophages for 2, 24, 48, and 72 hour incubation periods. Cells were then washed with PBS three times, fixed with fixative solution (1%(v/v) glutaraldehyde,

1mM magnesium chloride, 0.1 M sodium phosphate pH 7.3), using 1 ml fixative per well for 5minutes at 4°C. The fixative solution was then aspirated. The macrophages were monitored with an inverted microscope. Images were taken using an inverted or fluorescent microscope, where appropriate.

5.2.3. Animal Studies

Female C3H mice were obtained from Charles River at 6-7 weeks of age and then allowed to acclimatise for a further week prior to experiments. Before implanting murine fibrosarcoma RIF-1 cells (Twentyman *et al.*, 1980) the fur from the lower half of the mouse back was removed using clippers. The RIF-1 cells were previously cultured in RPMI 1640 medium supplemented with 15% foetal calf serum, penicillin and streptomycin, and were not passaged more than 7 times prior to trypsinisation for implantation. On the day of implantation the mice were slightly anaesthetised using intraperitoneal Hypnorm (mix of fentanylcitrate 0.315 mg/ml and fluanisone 10 mg/ml, 0.05 ml per mouse). An injection of 0.05 ml PBS containing $2x10^5$ RIF-1 cells was made intradermally mid way along the back of the mouse (approximately 2 cm from the base of the tail). To obtain an acceptable tumour implant care was taken to ensure that the cell suspension left a spherical bump at the implant site, devoid of any tracks caused by the leakage of cell suspension on needle removal. Tumours were visible after approximately seven days and were ready for delivery experiments 12-14 days post-implantation. On the day of treatment the mice were weighed and the dimensions of the tumour recorded. The average tumour was approximately 6-7 mm in diameter. The mice were then randomly assigned to the different treatment cages.

Injections were made into the right hind leg muscle of each mouse and the implanted tumours. Tissues were harvested following the killing (cervical dislocation) of the mice on days 2 and 7. To facilitate intramuscular injection the mice were anaesthetised using a combination of Hypnovel (midazolam 5mg/ml) and Hypnorm (1ml of Hypnovel + 1 ml of Hypnorm + 2 ml sterile water, injection 0.1 ml intraperitoneal) and the right hind thigh shaved with an electric shaver. Muscle injections were made using a sterile Hamilton syringe fitted with a 31G1/2 needle and calibrated dosing clamp. This clamp insured that each mouse was injected with 20 µl of formulation. The needle was left in place for a short period to prevent formulation being expelled by the tissue. The same procedure was carried out for the dosing of implanted tumour. Each 20 µl injection contained 0.8 mg of DHSA microparticles either with FITC coupled to their surface or without. A second large experiment was carried using the same parameters, however on this occasion 20 µl injections contained either a) saline alone as the control or b) 0.8 mg of FITC labelled DHSA microparticles. The mice were killed and tissue was excised after 2 and 24 hours after the injection.

5.2.3.1. Tissue Collection and Preparation

Specimens were frozen to cork blocks in Tissue-Tekk (Raymond A Lamb, UK) mounting medium by immersion in liquid nitrogen-cooled isopentane (BDH, UK). Subsequently 5 μm sections were cut using a Bright OTF/AS-001 motorized cryostat (Bright, Huntingdon, UK) maintained at –25 °C during sectioning.

5.2.4. Tissue Analysis

The tissues were analysed for the whereabouts of the FITC labelled microparticles in the tumour and muscle tissue. In addition, investigations were made on the influence of the particle injection on the immune response in tumour and muscle tissue. This involved using immunocytochemistry and enzyme histochemistry to assess the macrophage content of the relative tissues. Various staining procedures were used to observe the nuclear structure of the tissues. All reagents were purchased from BDH, UK except where stated.

5.2.4.1. Harris's Haematoxylin

This is a useful general-purpose alum haematoxylin and results in particularly clear nuclear staining. It stains nuceli a red colour, which is converted to the familiar blue-black when the section is washed in weak alkali (water). For this purpose cut sections were air dried for 1 hour and then fixed in cold acetone (-20°C). The sections were then rehydrated in running water for 3 minutes. Once rehydrated the tissues were incubated in Harris's Haematoxylin (mercury free, 1 part in 3 parts water) for 5minutes, then placed back in the water for a further 5 minutes. Sections were then incubated in

ACIDALCOHOL (1% of concentrated HCL in 100% IMS) for 1 minute and then returned to water for 5 minutes. The next incubation was in Eosin for 30 seconds and then water for 5 minutes. Finally, the tissue sections were incubated in IMS for 30 seconds twice, immersed into xylene for 30 seconds, and then into a final xylene incubation ready for mounting. DPX was used to mount the cover-slips.

5.2.4.2. Immunocytochemical staining (Avidin-Biotin Technique)

This method relies on the marked affinity of the glycoprotein avidin for biotin, a low molecular weight vitamin. Vectastain have developed a procedure called the 'ABC' or preformed complex method, which employs biotinylated antibody and a preformed 'Avidin:Biotinylated enzyme Complex', based on the affinity of avidin for biotin which can be used to amply the sensitivity of immunohistochemical staining.

Cut sections were left to air dry for at least 1 hour and 30 minutes, they are then fixed in cold acetone (-20°C) for 30 seconds. Sections then underwent two 5 minute washes in PBS. After washing sections were bathed for 20 minutes in incubation buffer (10 ml PBS containing 5 mg BSA and 2 drops of goat serum provided as Vectastain ABC-AP™ kit). After incubation sections underwent 2x (5 minute) washes in PBS, with slight agitation. Next the primary antibody was added, made up in incubation buffer to the required concentration, 100 µl to each section, and sections were incubated overnight at 4°C. The 1° antibody was washed off with 2x 5 minute washes in PBS. Sections were incubated with 100 µl secondary antibody, made up in PBS to the required concentration for 30 minutes. The ABC reagent (2 drops of part A in 10 ml of PBS mix then 2 drops of part B mix of ABC-AP™ kit) was prepared at this time. 2x 5 minute washes were followed by incubating sections in ABC reagent (100 µl per

section) for 30 minutes. This was followed by 2x 5 minute washes in PBS and incubation in Fast Red (Sigma Diagnostics, UK) for 8-10 minutes. Sections were then washed in running water, and counter stained with Mayers Haemotoxylin (Sigma Diagnostics, UK) for 1 minute, washed in cold running water, and mounted with Aquamount.

5.2.4.2.1. Antibodies

The 1° antibodies used were CD11b (Mac-1) (rat antimouse) a macrophage marker, and CD11c (hamster antimouse) a dendritic cell marker. 2° antibodies used were IgG:biotin goat anti rat (for CD11b) and IgG:biotin goat antihamster (for CD11c). All antibodies were purchased from Serotec, UK.

5.2.4.3. Enzyme Histochemistry (Non-Specifc Esterase: α Naphthyl Acetate Method)

Solutions

0.2M Sodium Phosphate	In 200ml of milli-Q water (stored at room temperature).
4% Pararosaniline HCL	0.5 g pararosaniline was dissolved in 10 ml milli-Q water, heated gently on hot plate (without boiling). 2.5 ml concentrated 12M HCL was added, cooled to room temperature, filtered and stored at 4°C.
4% Sodium Nitrite	In 12.5 ml of milli-Q water was stored at 4°C.
"Azotized Paraosaniline"	This was prepared freshly for each stain. 4% Parasaniline-HCL (0.4 ml) was added to 4% Sodium nitrite (0.4 ml) (solution was amber in colour).

Staining Solution

α Naphthyl acetate 3-5 mg Acetone 0.75 ml 0.2M Sodium phosphate 12.5 ml Azotized Pararosaniline	Prepared freshly for each stain. α naphthyl acetate was dissolved in acetone, mixed well, then sodium phosphate buffer was added (solution may become cloudy-OK). The solution was mixed well, and freshly prepared azotized pararosaniline was added with mixing prior to use.

Esterases are enzymes, which are capable of hydrolysing carboxylic acid. The majority of esterase enzymes are able to hydrolyse α naphthyl acetate as a substrate; these enzymes are called 'non-specific' esterases. The α naphthyl acetate method employed α naphthyl acetate as the substrate; the enzyme released α naphthol during hydrolysis of the substrate. The α naphthol was then coupled with a suitable diazonium salt to produce an insoluble azo dye at the site of enzyme activity, the diazonium salt used in this case was hexazotized pararosaniline, which gave good localisation of the enzyme.

Cut sections were air dried for at least 1 hour 30 minutes. Sections were fixed in Formol-Saline and rehydrated in running water. Sections were placed in freshly prepared staining solution (when staining solution is gold to orange in colour) for 5 minutes. To stop reaction sections were placed in running water for several minutes. Sections were dehydrated in 2x 100% IMS, transferred immediately to xylene 2x to clear, then mounted in DPX.

5.2.4.4. Imaging

All images in this chapter were obtained from inverted or normal Zeiss Microscopes, Zeiss, West Germany, or the Nikon Fluorescent Microscope, Nikon, Japan, using an Olympus OM4-Ti, 35 mm camera or Zeiss KS 300 version 3.0 where appropriate.

5.2.4.5. Data Analysis

The optimum number of sections per animal and number of fields per section were determined using 10 fields from each of 10 consecutive sections of RIF-1 tumour model. The cumulative number of fields that gave the lowest coefficient of variation was chosen as the number of fields per section to be quantified. The number of sections per tumour model was selected to give a SE ±25% of the mean (appendix *E*). One and two tailed t-tests were carried out to assess the statistical significance of the results.

5.3. RESULTS

5.3.1. SEM Images

The surface morphology of the FITC labelled DHSA microparticles was examined using scanning electron microscopy. Typical images of FITC labelled DHSA microparticles are shown in Figure 5.1. The microparticles appeared to have smooth surfaces with some particles containing internal voidages. The particles appear to be quite aggregated. The crystal fragments present in the images are the mannitol fragments in which the microparticles were blended to increase bulk density.

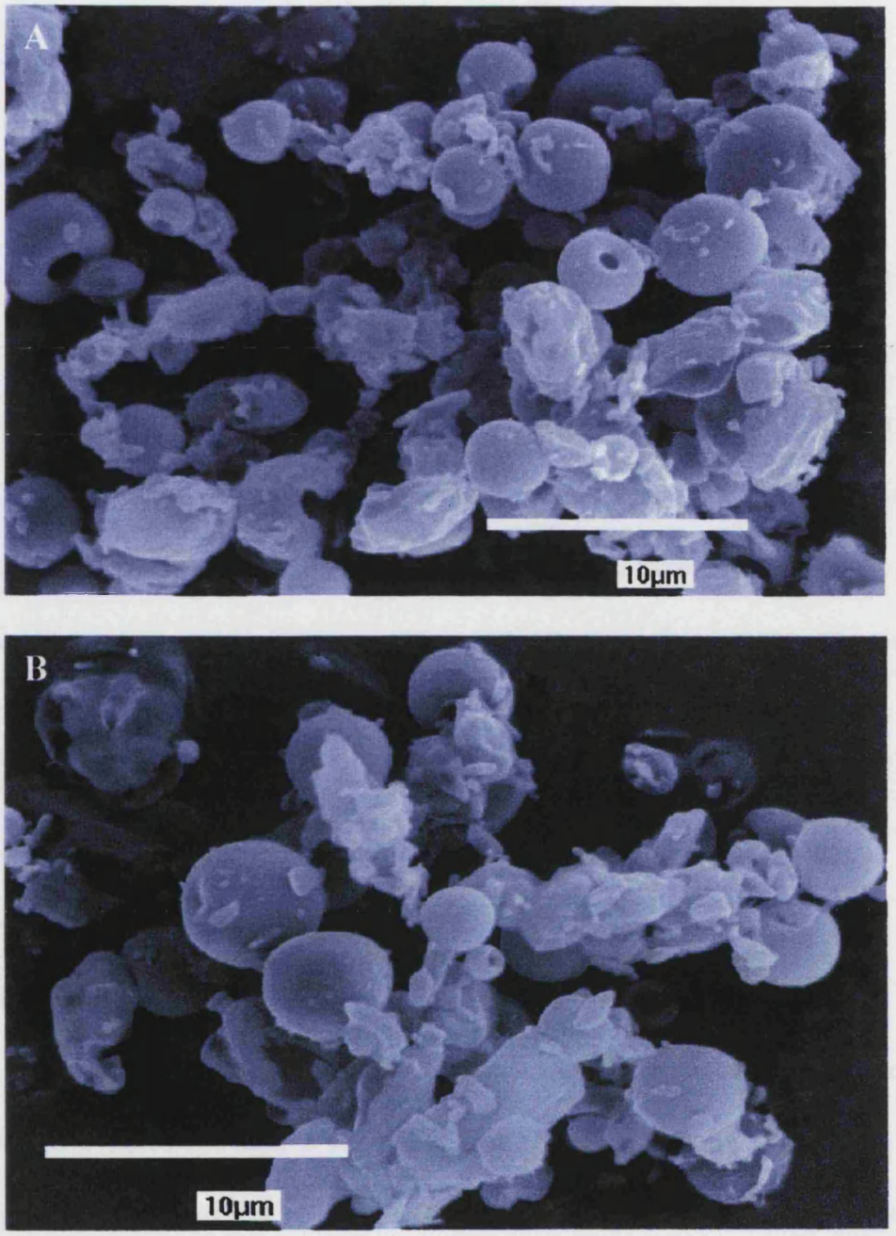


Figure 5.1. A and B are typical scanning electron microscopy images of spray dried defatted human serum albumin microparticles labelled with FITC.

5.3.2. Incubation of DHSA Microparticles with Macrophages *In Vitro*

FITC labelled DHSA microparticles were incubated *in vitro* with mouse peritoneal macrophages. 1.5 mg samples of aggregated or sonicated FITC labelled DHSA microparticles were incubated with mouse peritoneal macrophages in six well plates with RPMI medium at 37 °C. Samples were incubated at time intervals of 2, 24, 48, and 72 hours. Macrophage activity was monitored using an inverted light microscope. Cells were fixed to allow images to be taken where appropriate at x100, x200, and x600 magnification, using both normal/fluorescent and inverted light microscopes. Figure 5.2 shows typical images taken using inverted light microscopes of mouse peritoneal macrophages, after 2 hours of incubation at x200 magnification. It appeared that the phagocytic cells were beginning to exhibit dendritic morphology, which is very evident in Figure 5.3, which shows macrophages after 24 hours of incubation.

Images of sonicated particles incubated with mouse peritoneal macrophages are shown in Figures 5.4 and 5.5. The particles were of comparable size to peritoneal macrophages *in vitro*. It appears that microparticles incubated with macrophages at 2 hours (Figure 5.4 A) were under going the initial contact phase of phagocytosis, which was beginning to lead to the ingestion phase. At 24 hours of incubation (Figure 5.4 B) of microparticles with macrophages it appears that the majority of the cells were in the ingestion phase of phagocytosis. These cells were also becoming more dendritic. After 48 hours (Figure 5.5 A) incubation, the cells appeared to be in the digestion phase of phagocytosis i.e. the ingested particles were under going digestion, and after 72 hours it appeared that the majority of cells were in the digestion phase and were beginning to

look unhealthy. Some of the phagocytic cells may have completed digestion, as the cells appear to have a glowing halo, which could be attributed to the FITC.

Figures 5.6 and 5.7 show images of aggregated microparticles were introduced to peritoneal macrophages. It appeared that at 2 hours of incubation (Figure 5.6) only a small number of cells that had microparticles bound to their surface. In fact the aggregate of microparticles had a combined size which was greater than the phagocytic cells. By 72 hours the cells had ingested some free microparticles or small aggregates and were under going the digestion phase. The larger aggregated microparticles were still not ingested after 72 hours.

The phagocytosis of the microparticles was also monitored using fluorescence microscopy. Figure 5.8 shows untreated mouse peritoneal macrophages at x200 magnification observed by inverted light microscopy (Figure 5.8 A) and normal light settings at x100 magnification (Figure 5.8 B) on a fluorescence microscope. The morphology of the cells is clearer in the Figure 5.8 A but by using normal light the cells could also be clearly visualised using the fluorescence microscope. As expected at this sensitivity no fluorescence was observed from the cells. Figures 5.9-5.12 show further evidence of the three phases of phagocytosis. At 2 hour incubation (Figure 5.9) most cells had associated fluorescence which may have been are undergoing the initial attachment phase. At 24 hours (Figure 5.10) the majority of the phagocytic cells were probably undergoing the ingestion phase and beginning the digestion phase. The spherical particles could be seen inside the cells by normal light microscopy (Figure 5.10 A). By 48 hours it appeared that many of the cells had under gone the digestion phase as the FITC was distributed throughout the main body of the cell and was starting to be seen in the dendritic parts of the cells. By 72 hours it clear that the macrophages had digested more of the particles, releasing FITC to the dendritic process of the cells.

Images taken at x600 magnification are shown in Figures 5.13-5.18. These images suggest that at 48 hours the FITC had not spread throughout the cell but by 72 hours FITC could be seen throughout the cytoplasm.

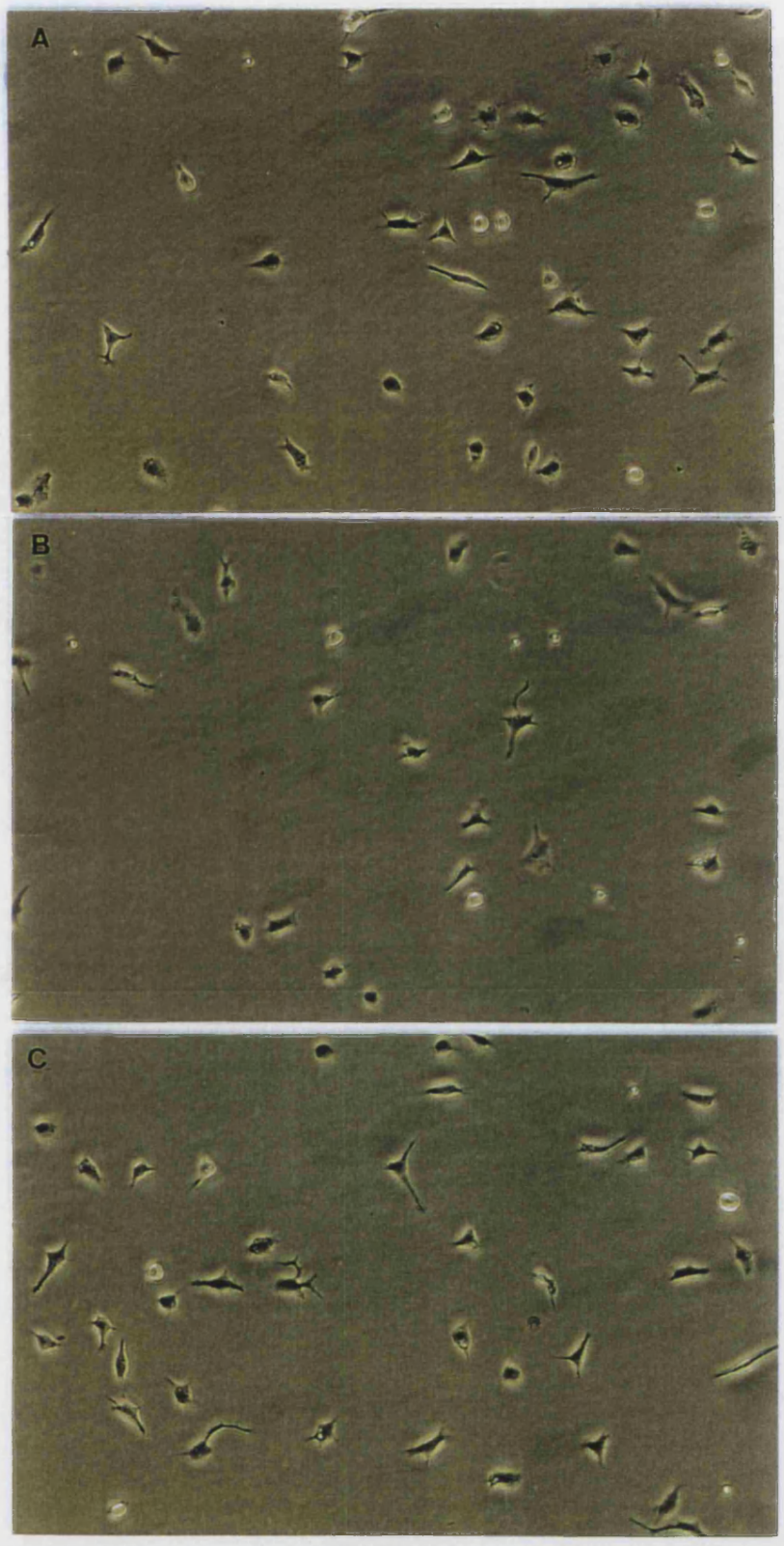


Figure 5.2. Typical images from inverted light microscopy of mouse peritoneal macrophages. A-C show typical images of untreated macrophages incubated with RPMI *in vitro*, after a 2 hour incubation period. Images were taken at x200 magnification.

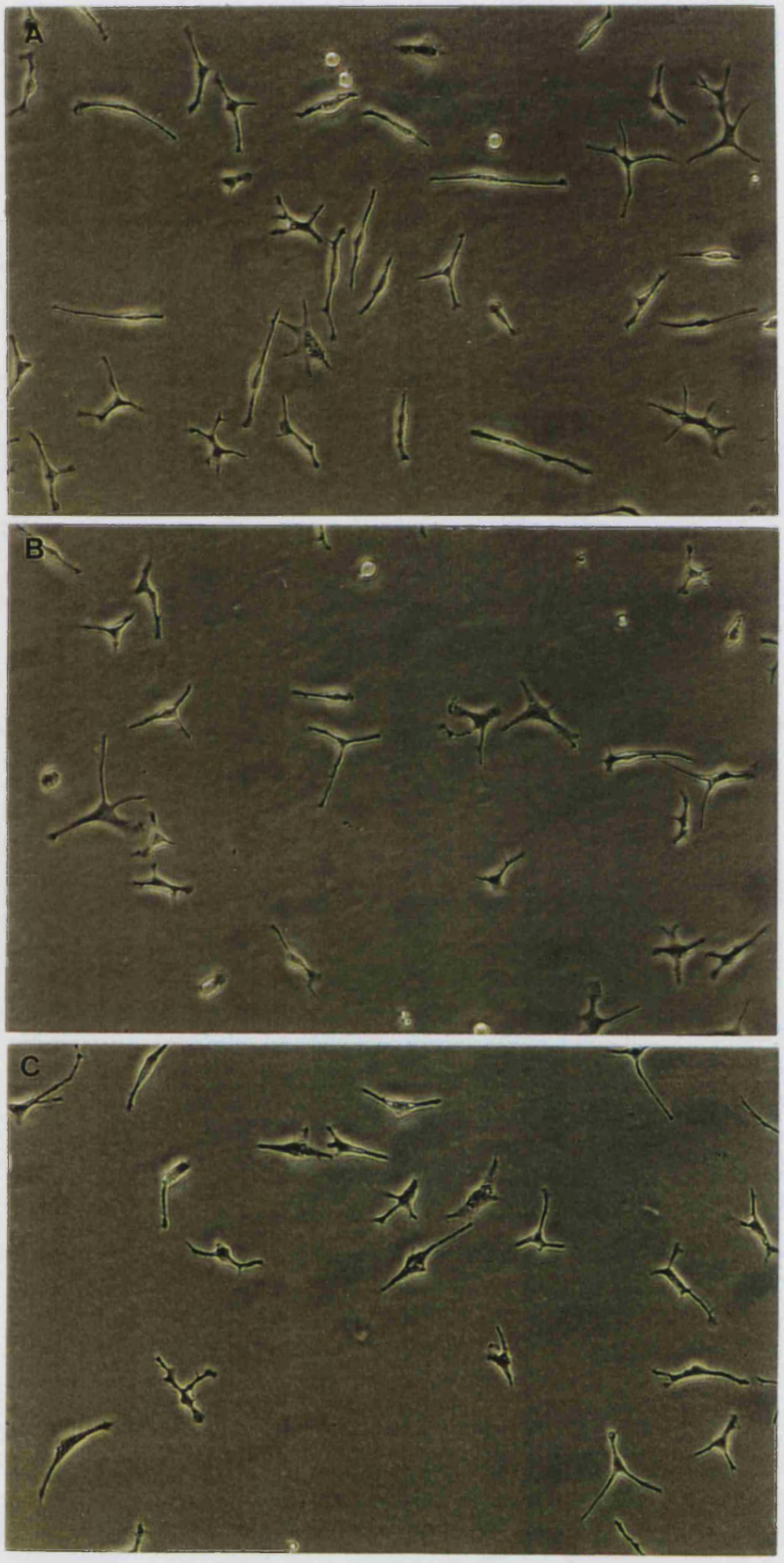


Figure 5.3. Mouse peritoneal macrophages. A-C show typical images of untreated macrophages incubated with RPMI for 24 hours. Images were taken at x200 magnification.

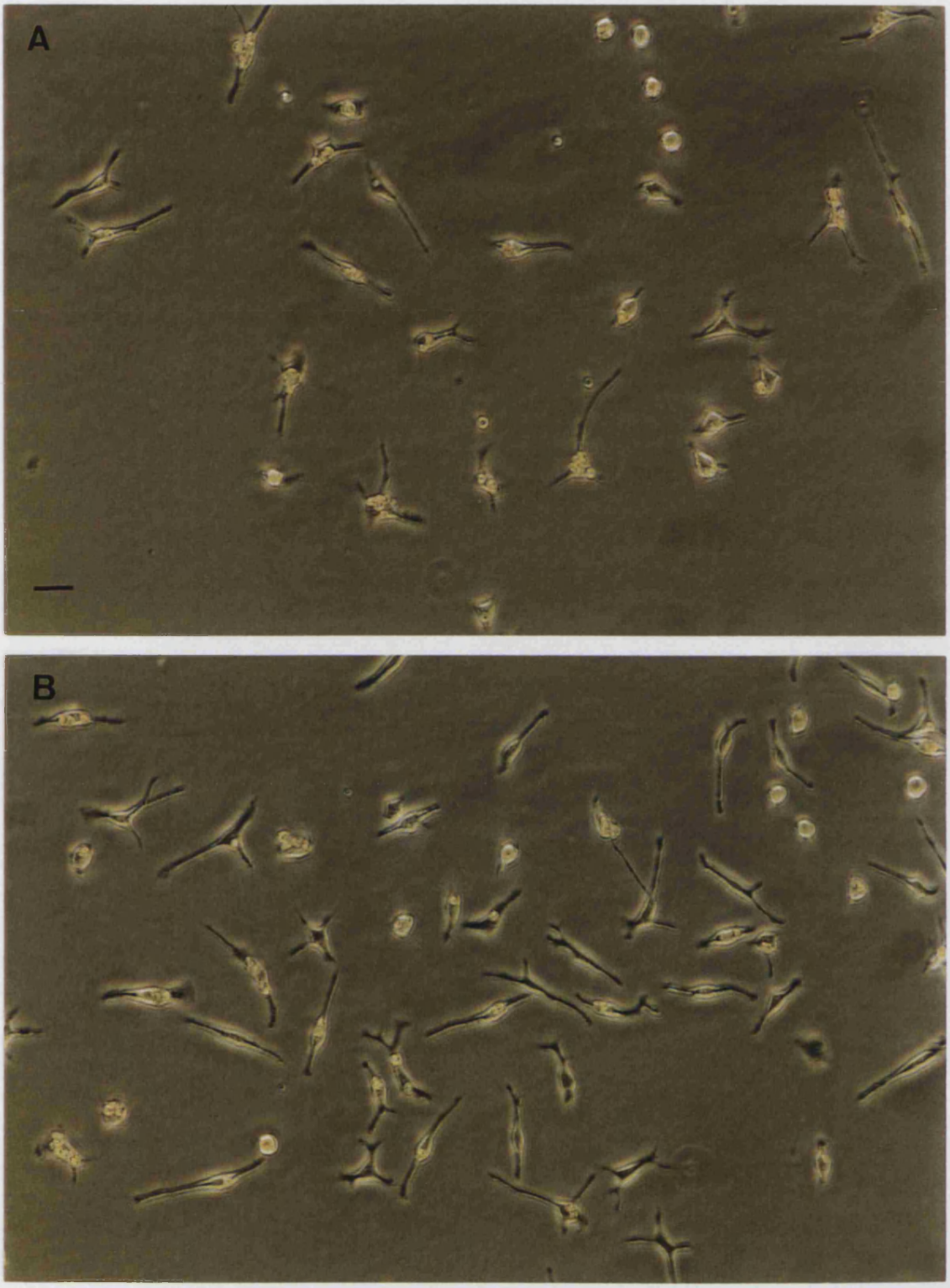


Figure 5.4. Mouse peritoneal macrophages treated with FITC labelled DHSA microparticles. Macrophages were treated with 1.5 mg samples of FITC labelled DHSA microparticles and incubated for 2 (A) and 24 (B) hour periods. Images were taken at x200 magnification. Scale bar = 100μm.

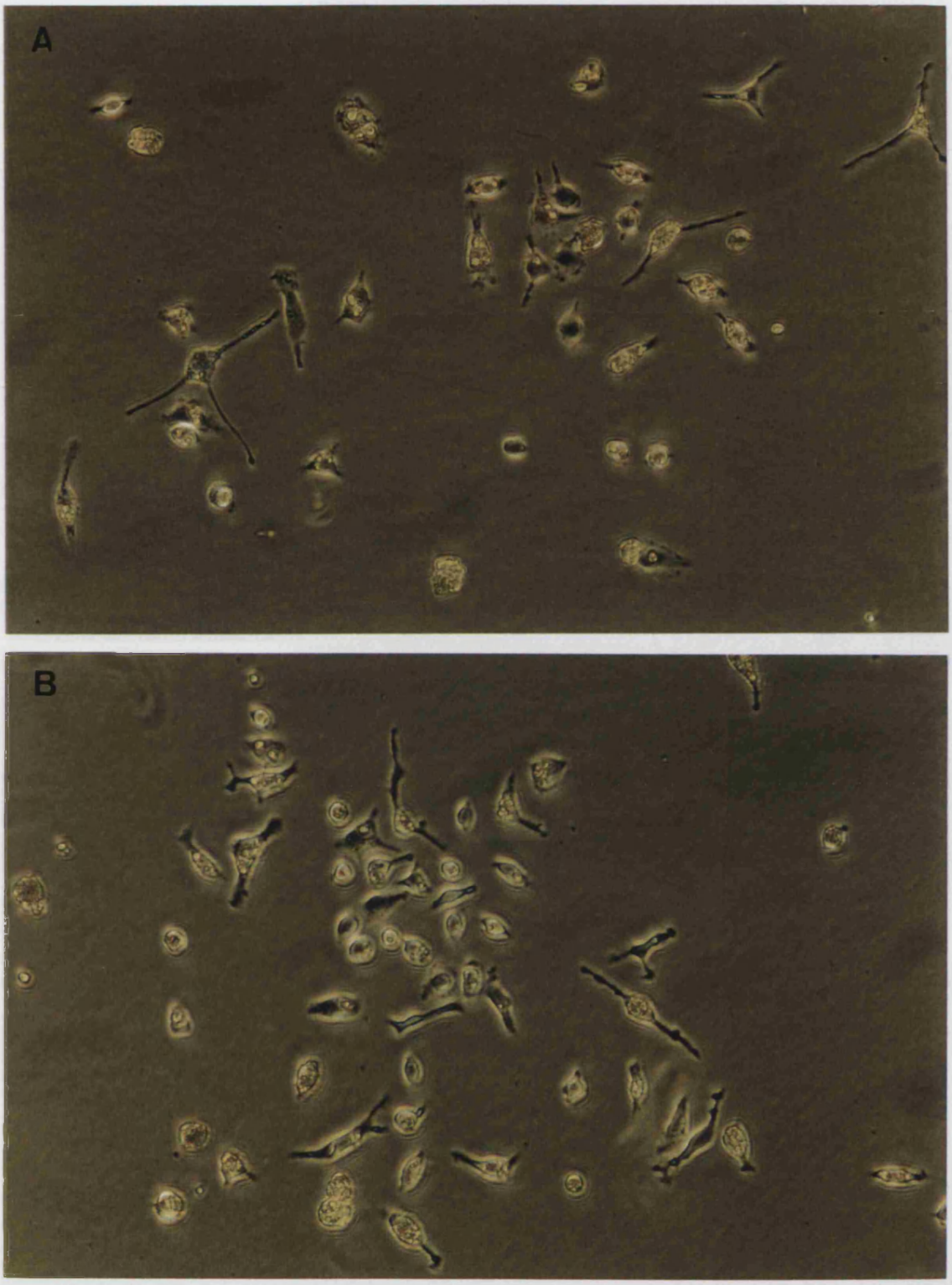


Figure 5.5. Mouse peritoneal macrophages treated with FITC labelled DHSA microparticles. Macrophages were treated with 1.5 mg of FITC labelled DHSA microparticles for 48 hour (A) and 72 hour (B) periods. Images were taken at x200 magnification.

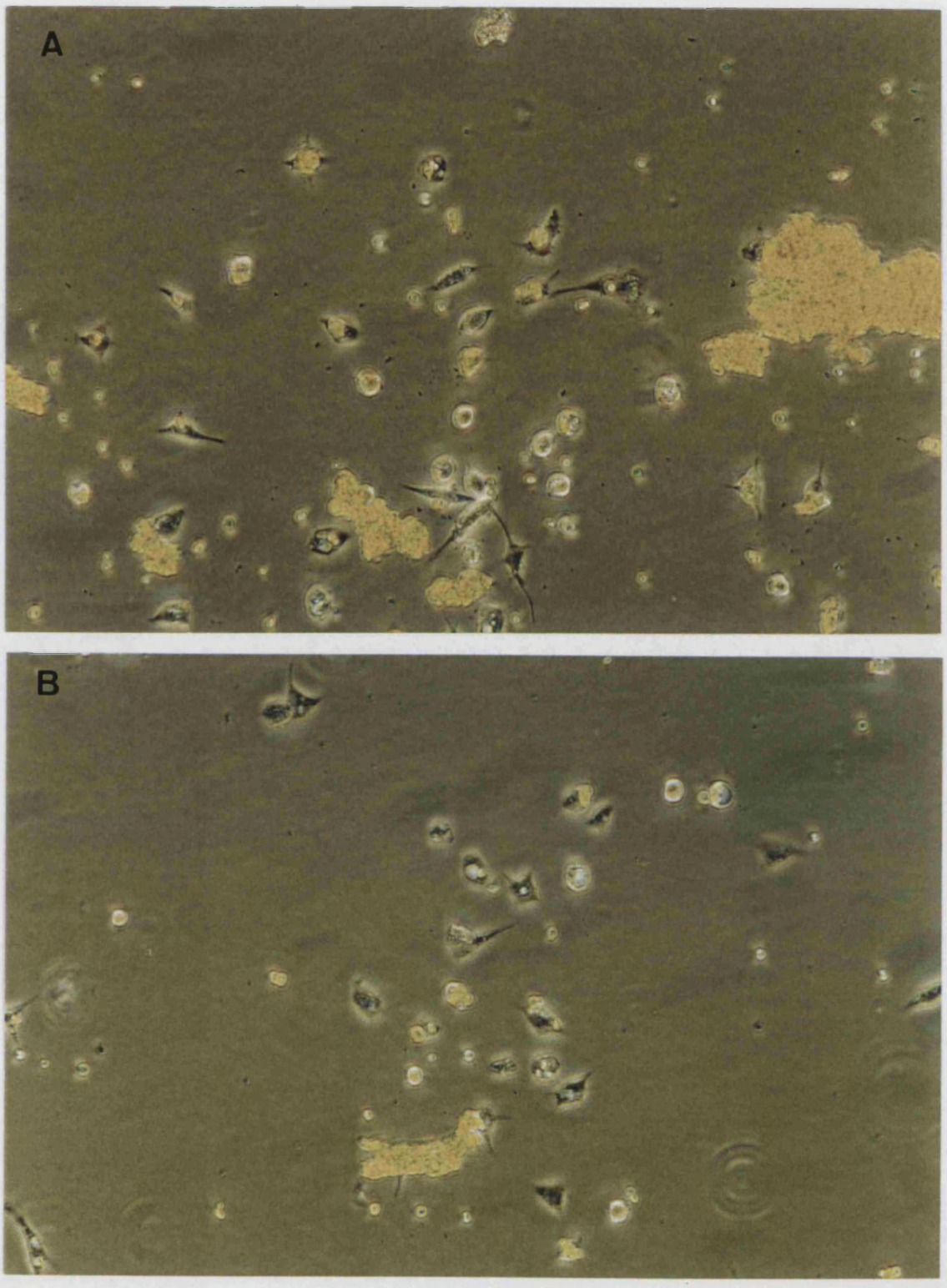


Figure 5.6. Mouse peritoneal macrophages treated with aggregated FITC labelled DHSA microparticles. Macrophages were treated with 1.5 mg of aggregated FITC labelled DHSA microparticles for 2 hours. Images were taken at x200 magnification.

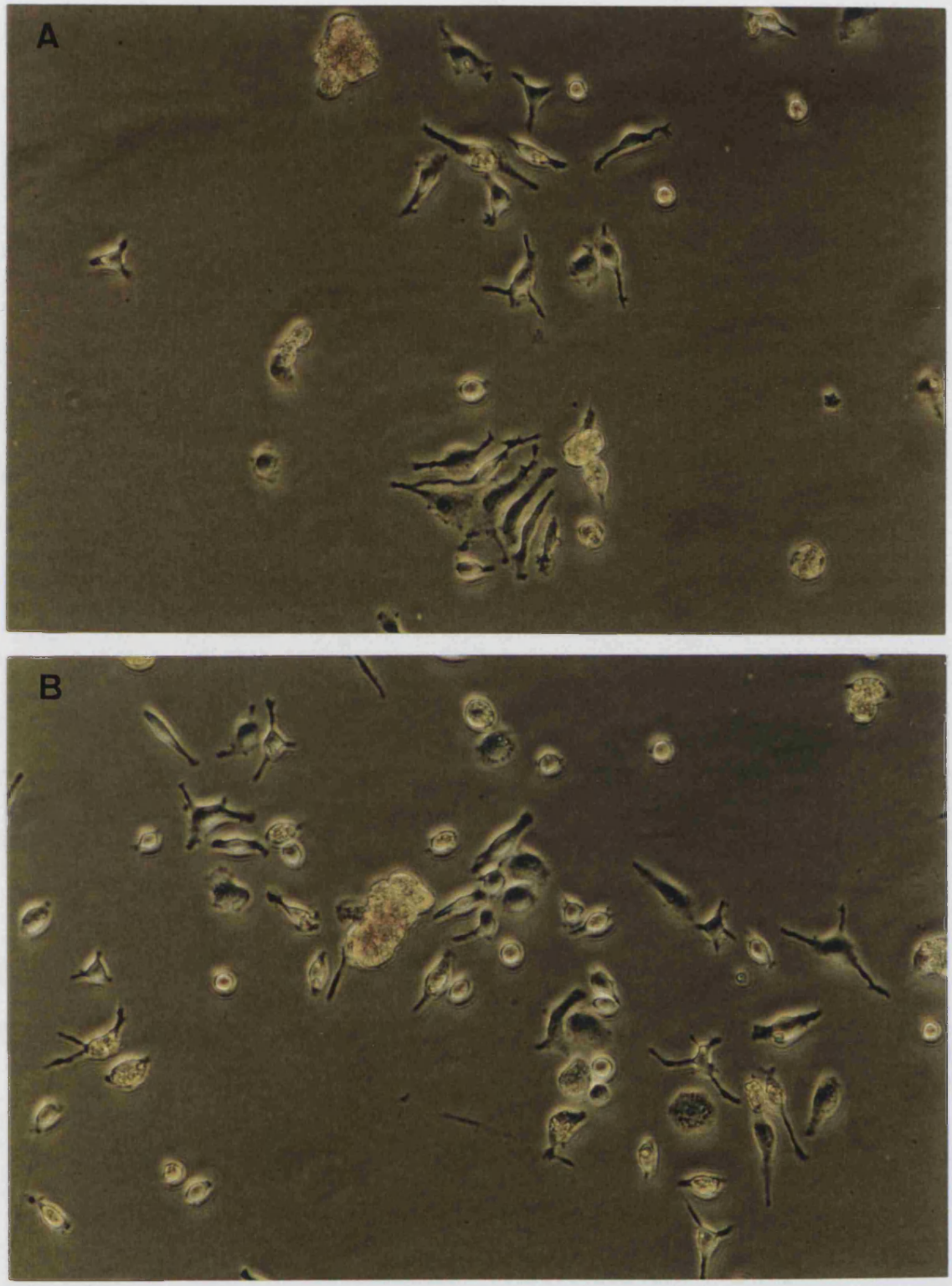


Figure 5.7. Mouse peritoneal macrophages treated with aggregated FITC labelled DHSA microparticles. Macrophages were treated with 1.5 mg of aggregated FITC labelled DHSA microparticles for 72 hours. Images were taken at x200 magnification.

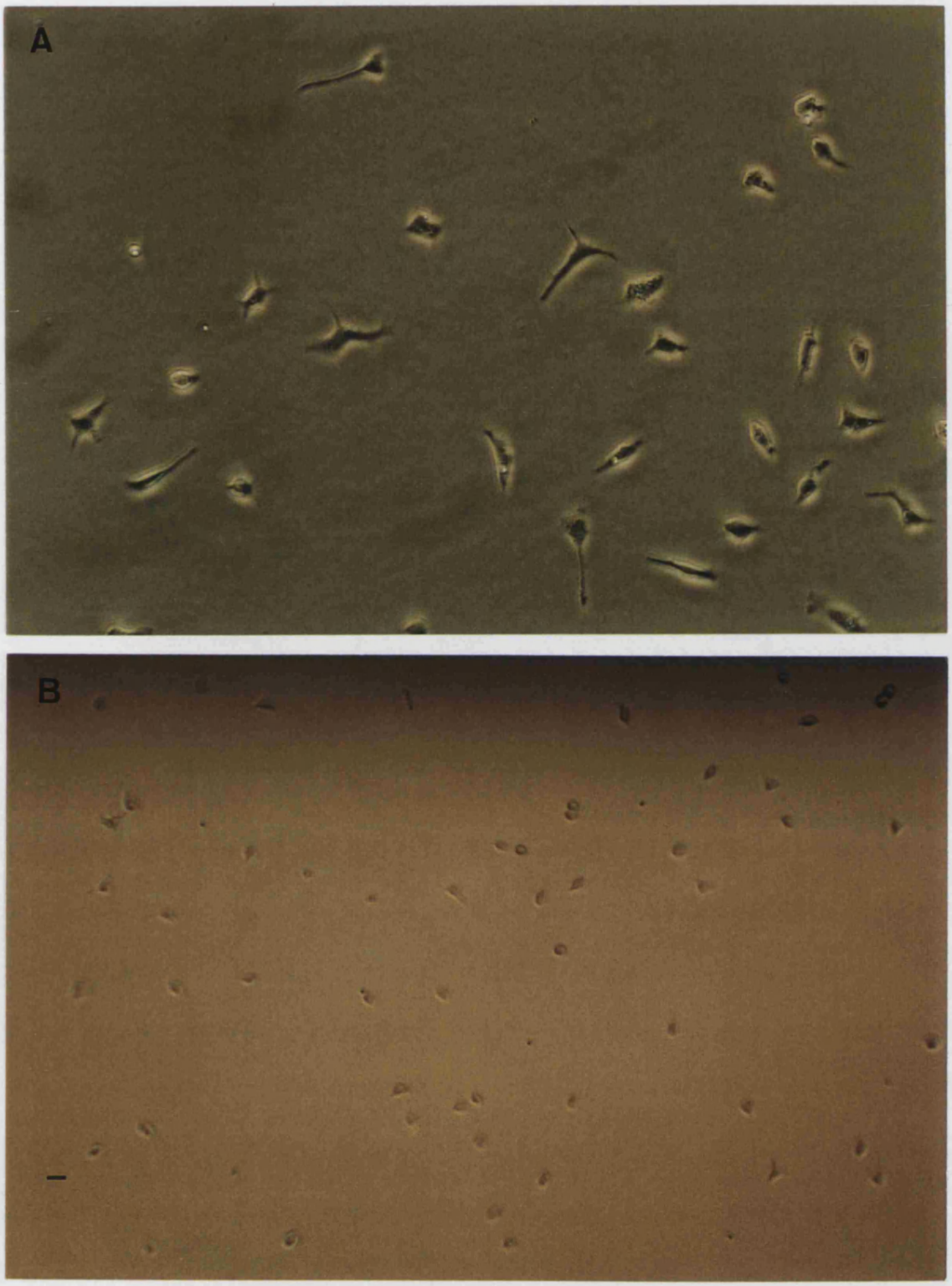


Figure 5.8. Typical images of mouse peritoneal macrophages obtained from inverted (A) and fluorescent (B) microscopes. Untreated macrophages at x200 magnification (A) and, x100 magnification (B). Scale bar = 100μm.

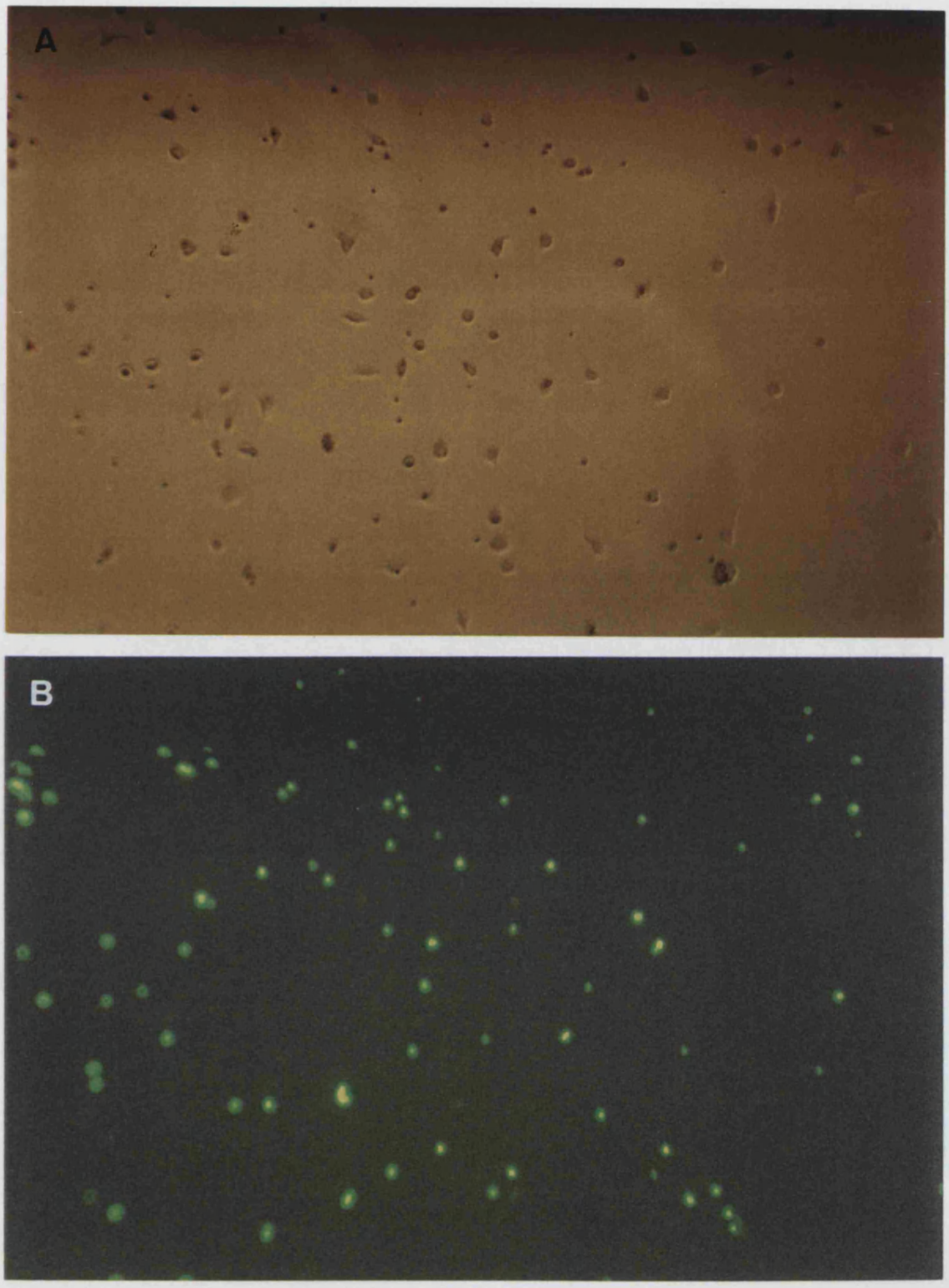


Figure 5.9. Normal light (A) and fluorescent (B) images of mouse peritoneal macrophages treated with FITC labelled DHSA microparticles. Macrophages were treated with sonicated FITC labelled DHSA microparticles for 2 hours.

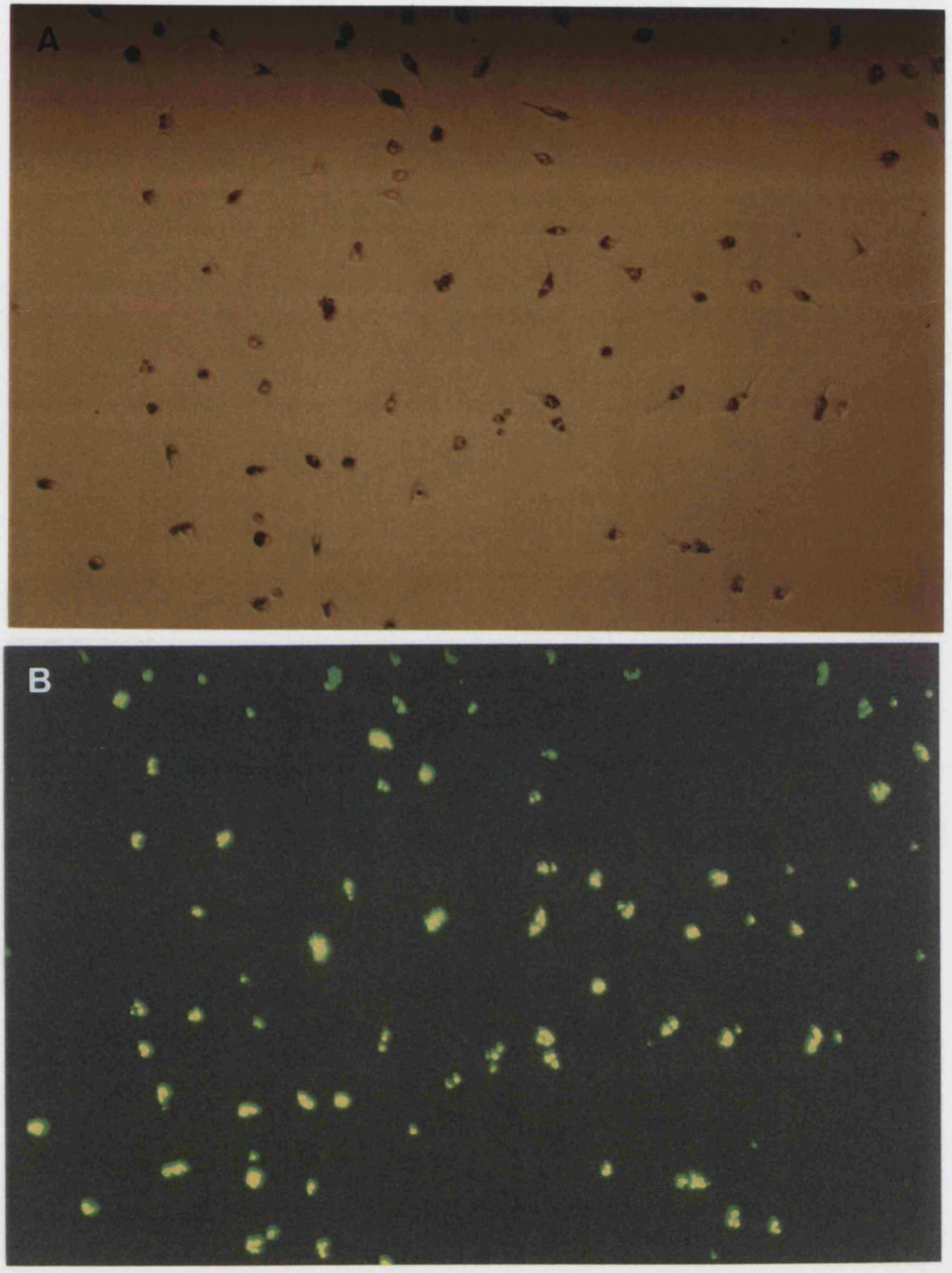


Figure 5.10. Mouse peritoneal macrophages treated with FITC labelled DHSA microparticles. Macrophages were treated with sonicated FITC labelled DHSA microparticles for 24 hours.

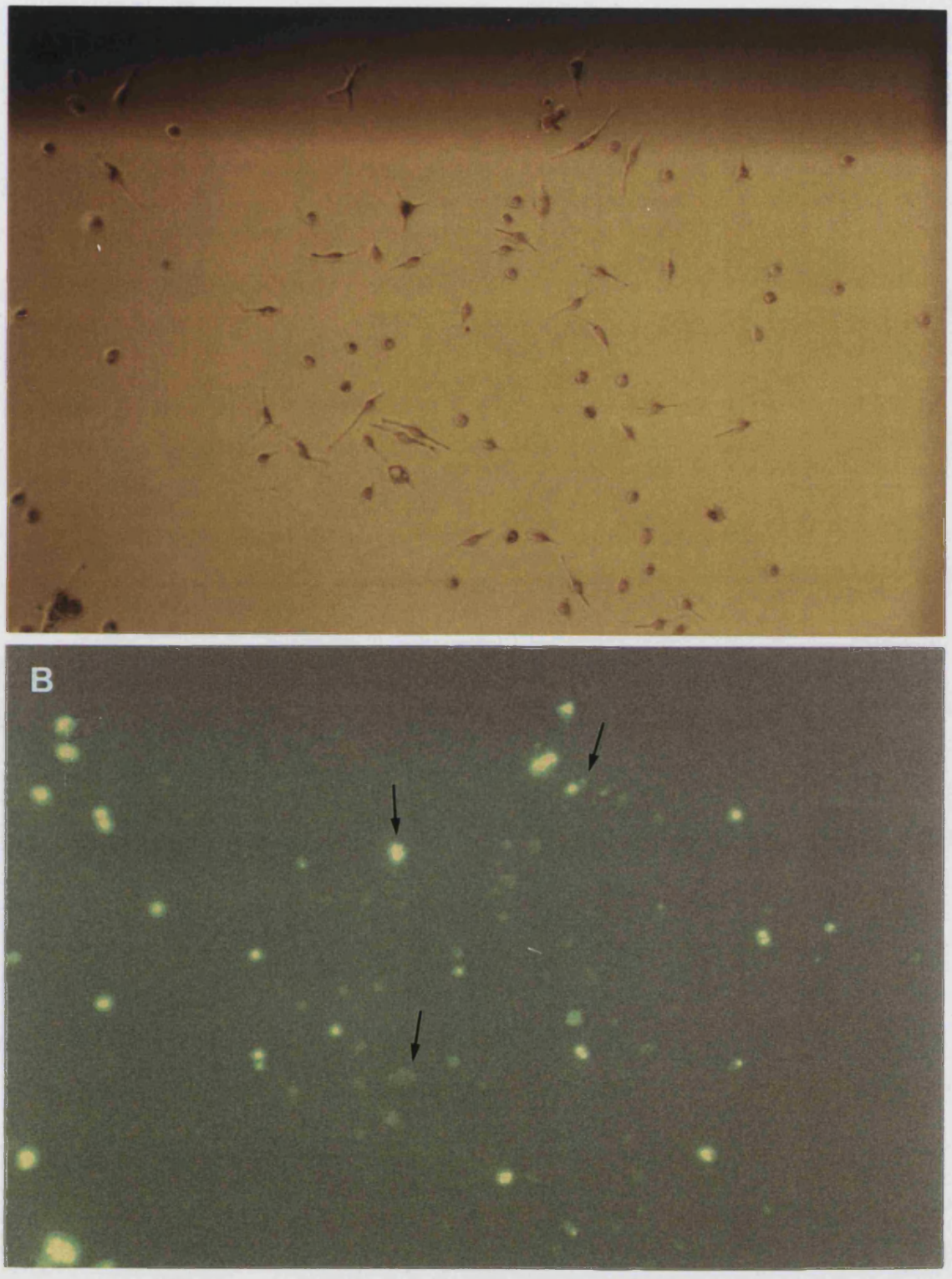


Figure 5.11. Mouse peritoneal macrophages treated with FITC labelled DHSA microparticles. Macrophages were treated with 1.5 mg of sonicated samples of FITC labelled DHSA microparticles for 48 hours. Images were taken at x100 magnification.

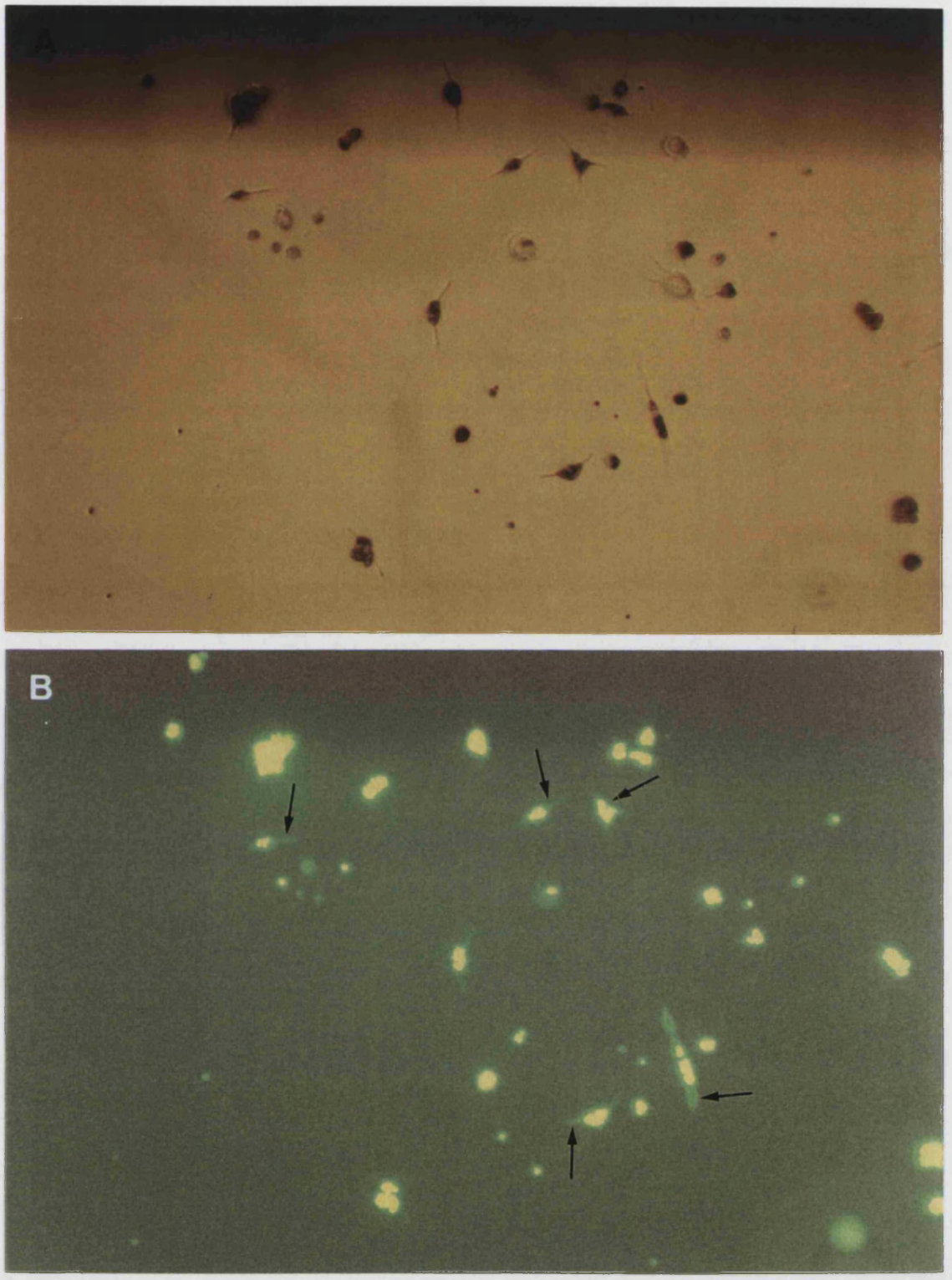


Figure 5.12. Mouse peritoneal macrophages treated with FITC labelled DHSA microparticles. Macrophages were treated with 1.5mg sonicated samples of FITC labelled DHSA microparticles for 72 hours.

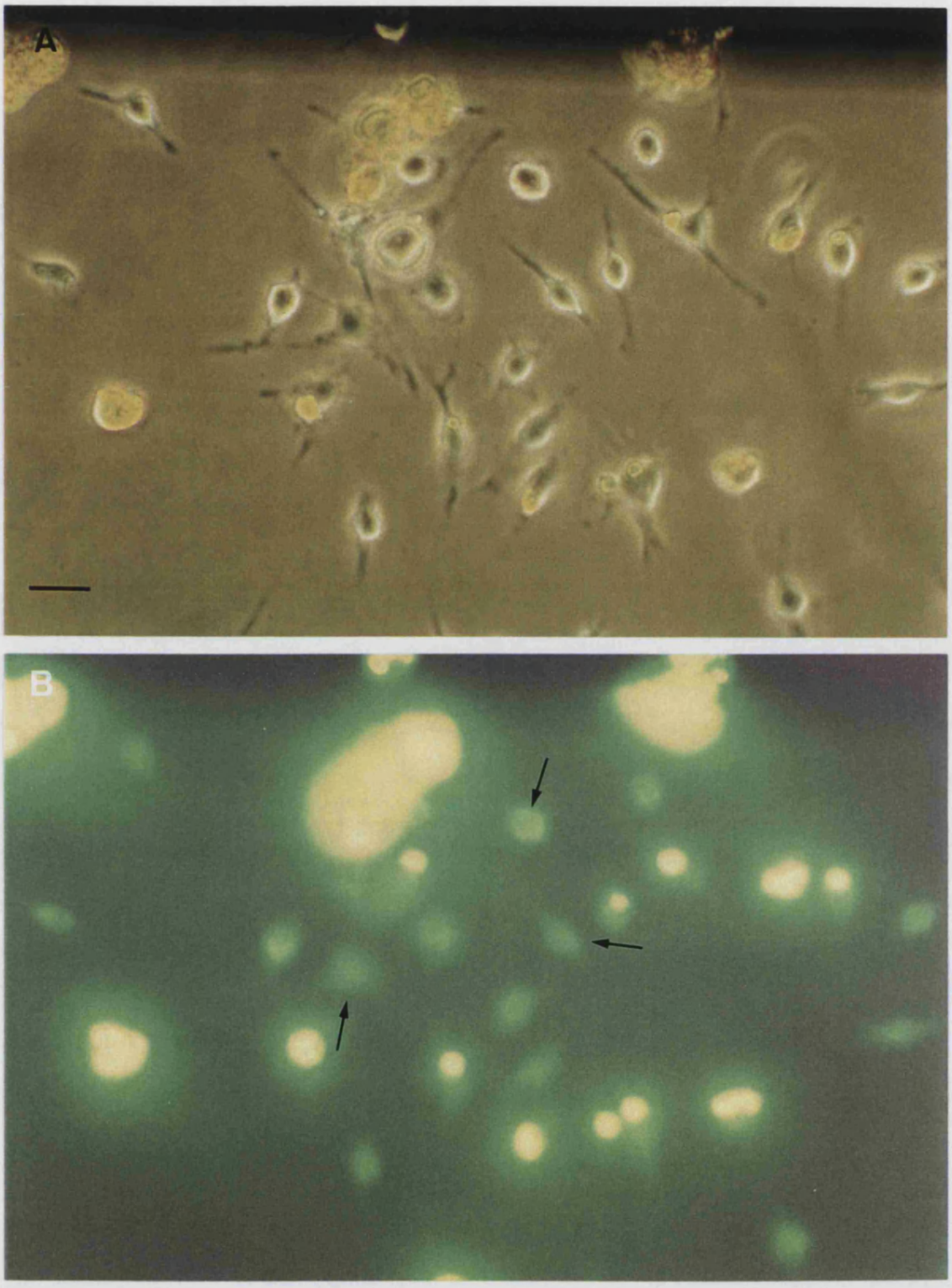


Figure 5.13. Mouse peritoneal macrophages treated with FITC labelled DHSA microparticles. Macrophages were treated with 1.5 mg of FITC labelled DHSA microparticles for 24 hours. Images were taken at x600 magnification. Scale bar = 100μm.

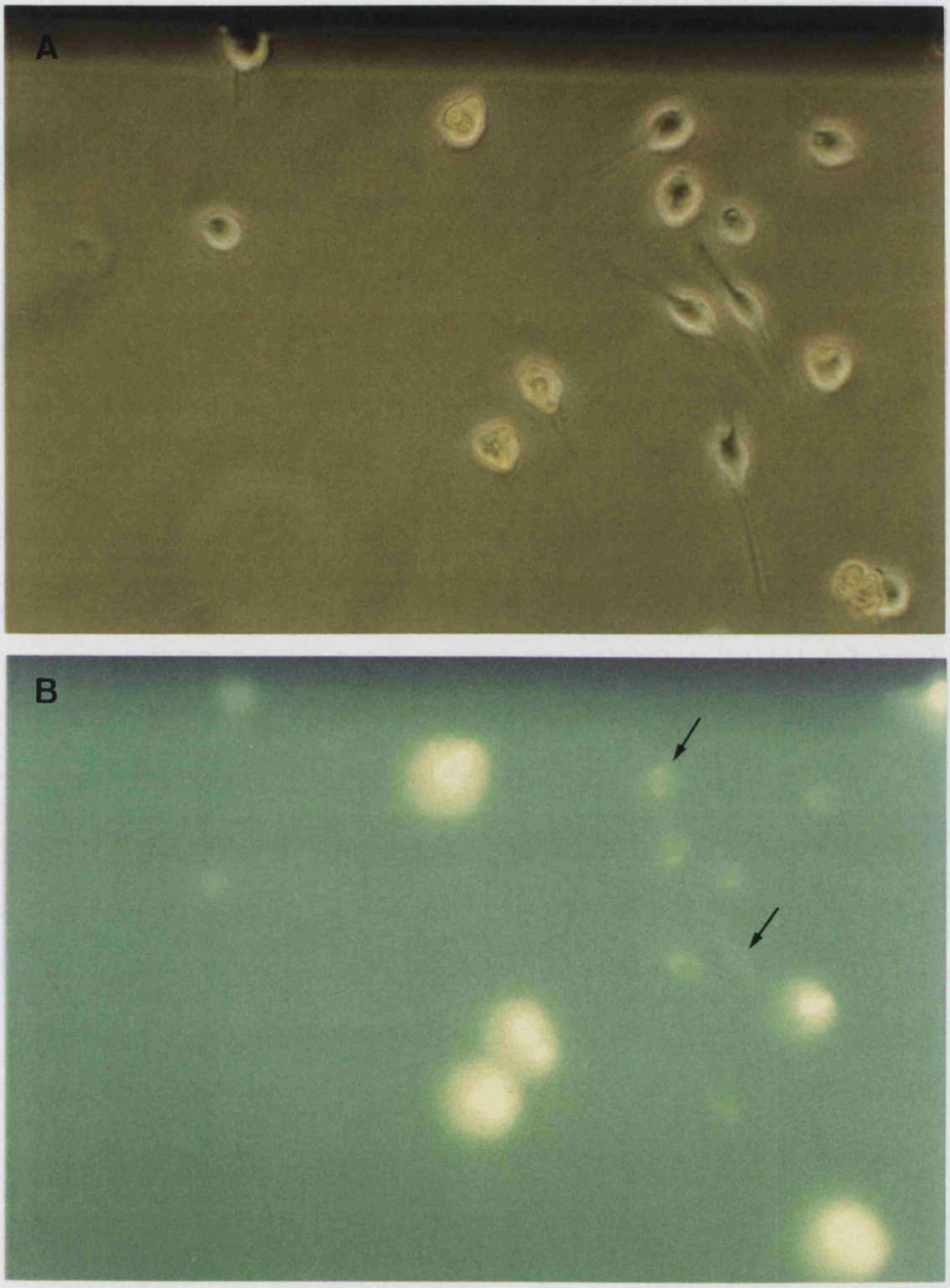


Figure 5.14. Mouse peritoneal macrophages treated with FITC labelled DHSA microparticles. Macrophages were treated with 1.5 mg of FITC labelled DHSA microparticles for 24 hours. Images were taken at x600 magnification.

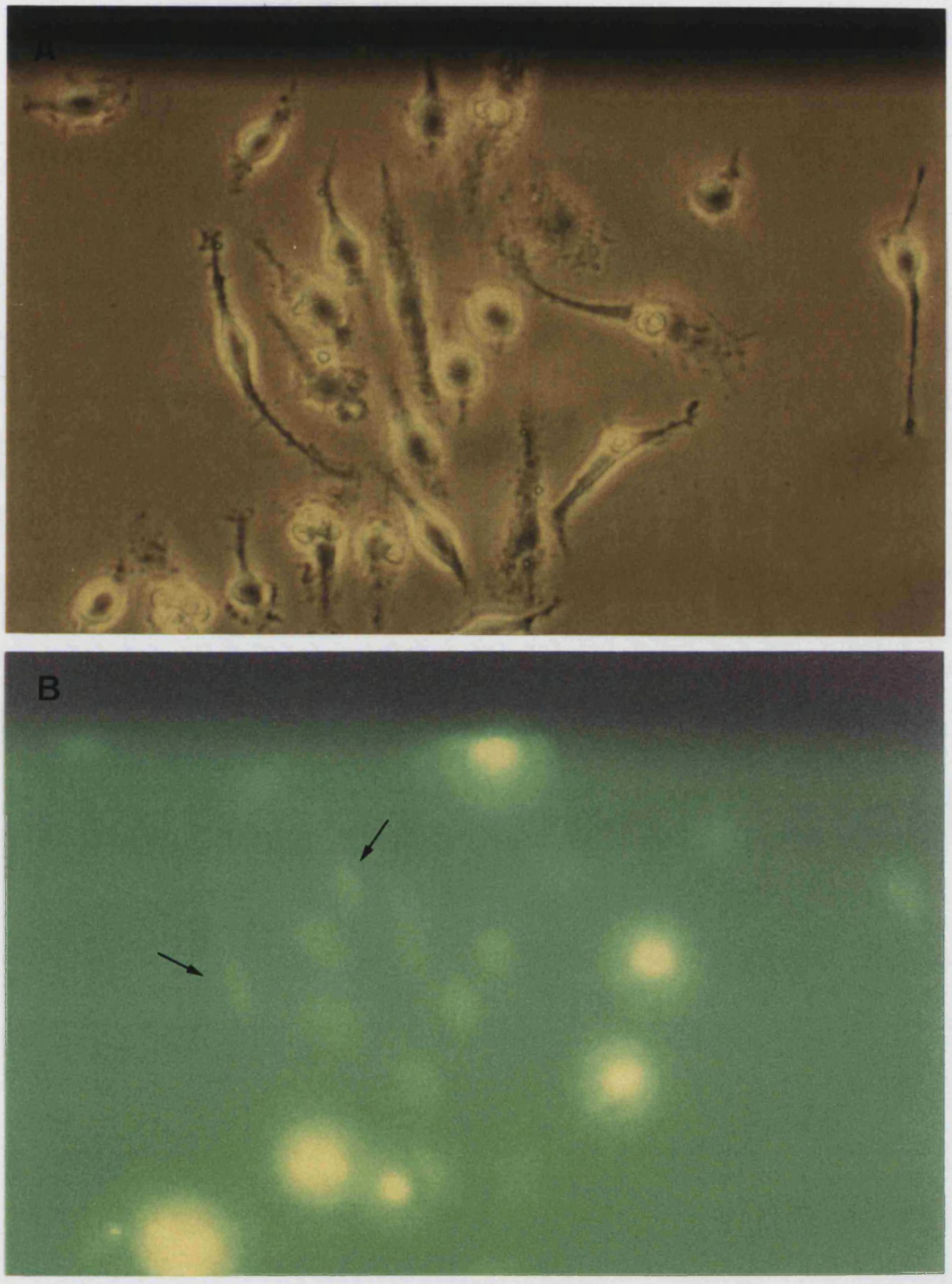


Figure 5.15. Mouse peritoneal macrophages treated with FITC labelled DHSA microparticles. Macrophages were treated with 1.5 mg of FITC labelled DHSA microparticles for 48 hours. Images were taken at x600 magnification.

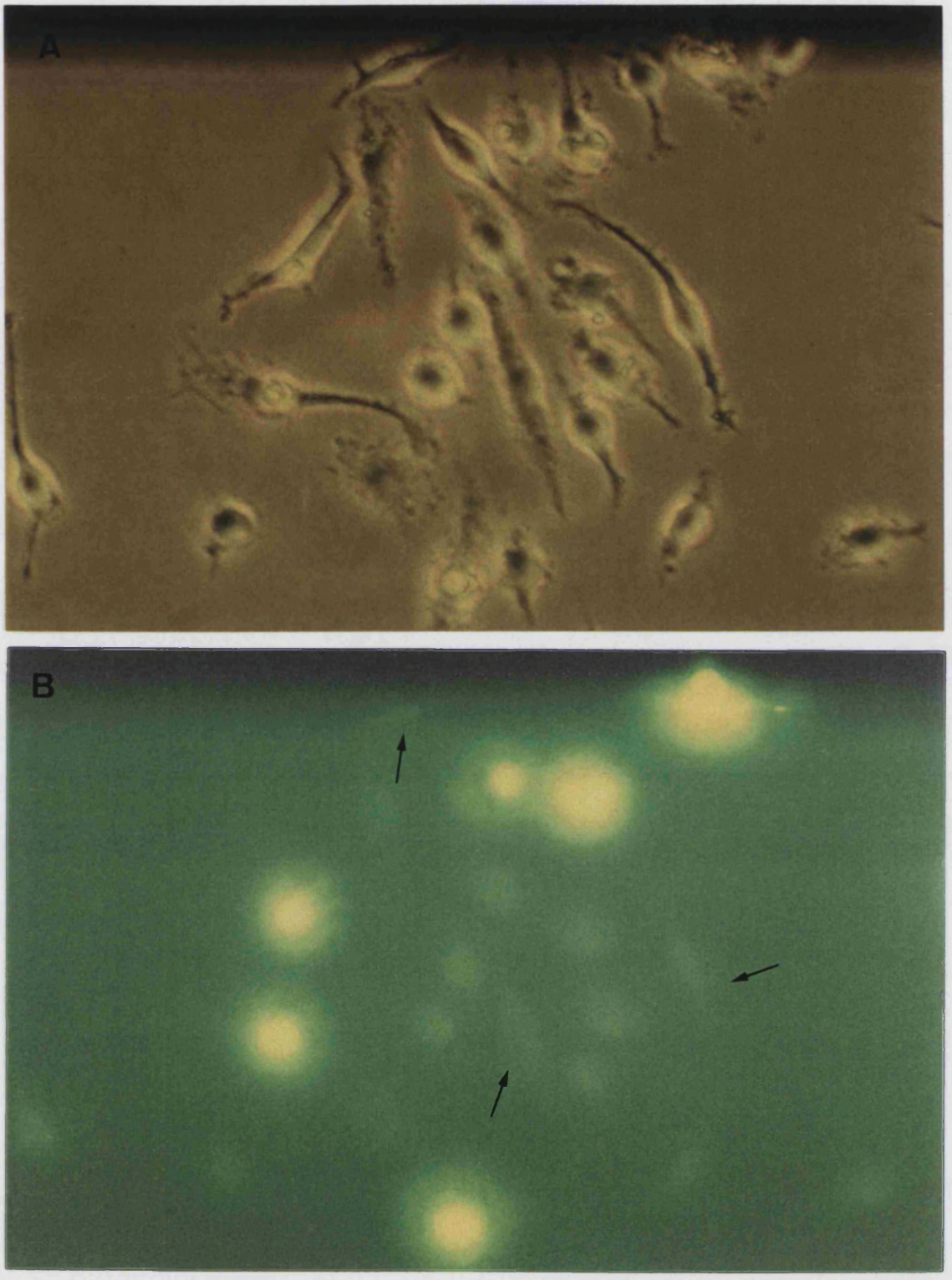


Figure 5.16. Mouse peritoneal macrophages treated with FITC labelled DHSA microparticles. Macrophages were treated with 1.5 mg of FITC labelled DHSA microparticles for 48 hours. Images were taken at x600 magnification.

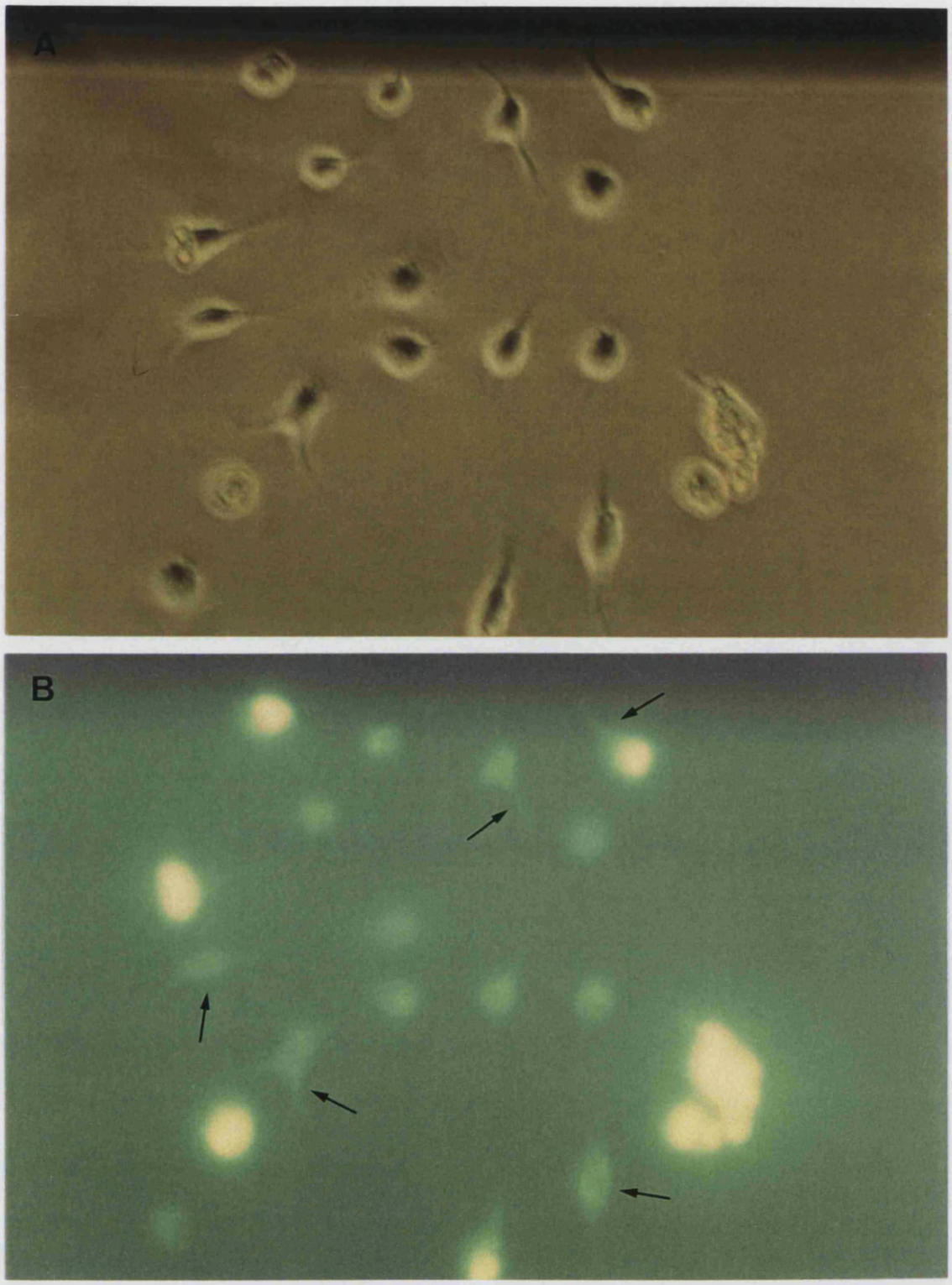


Figure 5.17. Mouse peritoneal macrophages treated with FITC labelled DHSA microparticles. Macrophages were treated with 1.5 mg of FITC labelled DHSA microparticles for 72 hours. Images were taken at x600 magnification.

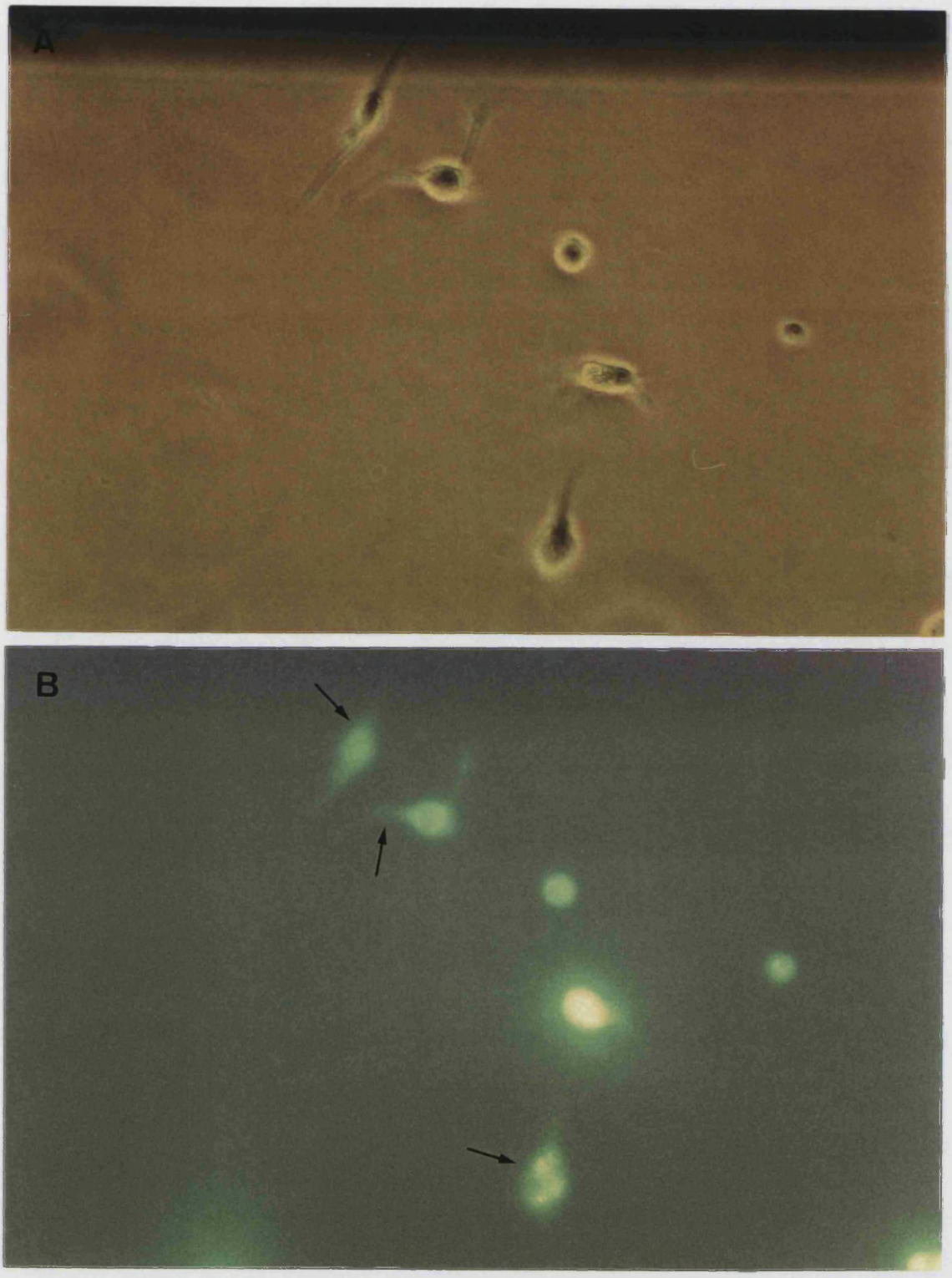


Figure 5.18. Mouse peritoneal macrophages treated with FITC labelled DHSA microparticles. Macrophages were treated with 1.5 mg of FITC labelled DHSA microparticles for 72 hours. Images were taken at x600 magnification.

5.3.3. *In Vivo* Evaluation of DHSA Microparticles

DHSA microparticles were administered directly to mouse muscle and tumour by injection. The fate of microparticles was investigated *in vivo* after 2 or 7 day periods; both FITC and non-FITC labelled microparticles were analysed. Tissues were analysed using enzyme histochemistry for evidence of macrophage infiltration, and by fluorescence microscopy to investigate microparticle distribution and stability.

Further *in vivo* experiments were carried out, directly administering FITC labelled DHSA microparticles to tumour tissue, and investigated their fate 2 and 24 hours after injection.

To assess tissue distribution of the FITC labelled microparticles, excised tumours were sectioned all the way through (from top to the bottom of the tumour) at 5 μm per section, and tissues were viewed using fluorescence microscopy. Figure 5.19 shows typical images obtained at 2 days after injection. The majority of microparticles were quite localised in their distribution in the tumour tissue (Figure 5.19 A). It also appeared that at day 2 the majority of the microparticles were still intact. As expected the microparticle distribution was localised within the middle region of the tumours near the site of injection. Tumours were also sectioned all the way through 7 days after injection (Figure 5.20). After this period microparticles were less easily detectable using fluorescence microscopy, and the more diffuse distribution of FITC suggested that they were partially degraded.

Macrophages in the excised tissue were identified through enzymehistochemistry using the non-specific esterase (α naphthyl acetate method) (Figures 5.23 and 5.24). Figure 5.21 D shows the nuclear structure of the tumour tissue, and Figure 5.21 F shows positive non-specific esterase staining of the tumour tissue

revealing macrophages in the tumour tissue. Non-specific esterase staining was also carried out on the excised mouse thigh muscle tissue (Figure 5.22) and as expected no macrophages were revealed in the tissue. However the test revealed the two different types of muscle fibre within the thigh muscle. Once the macrophages had been identified in tumour tissue, macrophage counts were carried out. The optimum number of sections per animal and the number of fields of view per section, were determined using 10 fields from each of the 10 consecutive sections of the RIF-1 tumour. The cumulative number of fields that gave the lowest coefficient variation, was chosen as the number of fields per section to be quantified, in this case it was 10, and the number of sections per tumour model was selected to be 5 which gave an S.E. ±25% of the mean (appendix *E*).

Figure 5.25 describes results from the first *in vivo* experiment, in which 1.5 mg of unlabelled and FITC labelled microparticles were injected directly. Tumours excised 2 days after treatment with FITC labelled microparticles appeared to have fewer macrophages than the control tumours, which were untreated. However, for tumours treated with unlabelled microparticles, after 7 days, there was no significant change in the number of macrophages present, in comparison to the untreated tumours. Tumours treated with FITC-labelled microparticles, which were excised 7 days after injection, appeared to have fewer macrophages than the untreated tumours, and slightly less than those tumours, which were excised after 2 days.

In a second larger *in vivo* experiment, tumours were either untreated, treated with saline or treated with FITC labelled microparticles (Figure 5.28), and excised either 2 or 24 hours after injection. Again fluorescence microscopy was used to observe the distribution of the microparticles with in the tumour. The microparticles appeared to be distributed around the periphery of the tumour (Figure 5.26). Injecting the

tumours with saline alone caused an increase in the number of macrophages present, 2 and 24 hours after injection. However, after 2 hours there appears to be a further increase in the number of macrophages present in tumours treated with FITC labelled microparticles, where as after 24 hours the number of macrophages present had decreased.

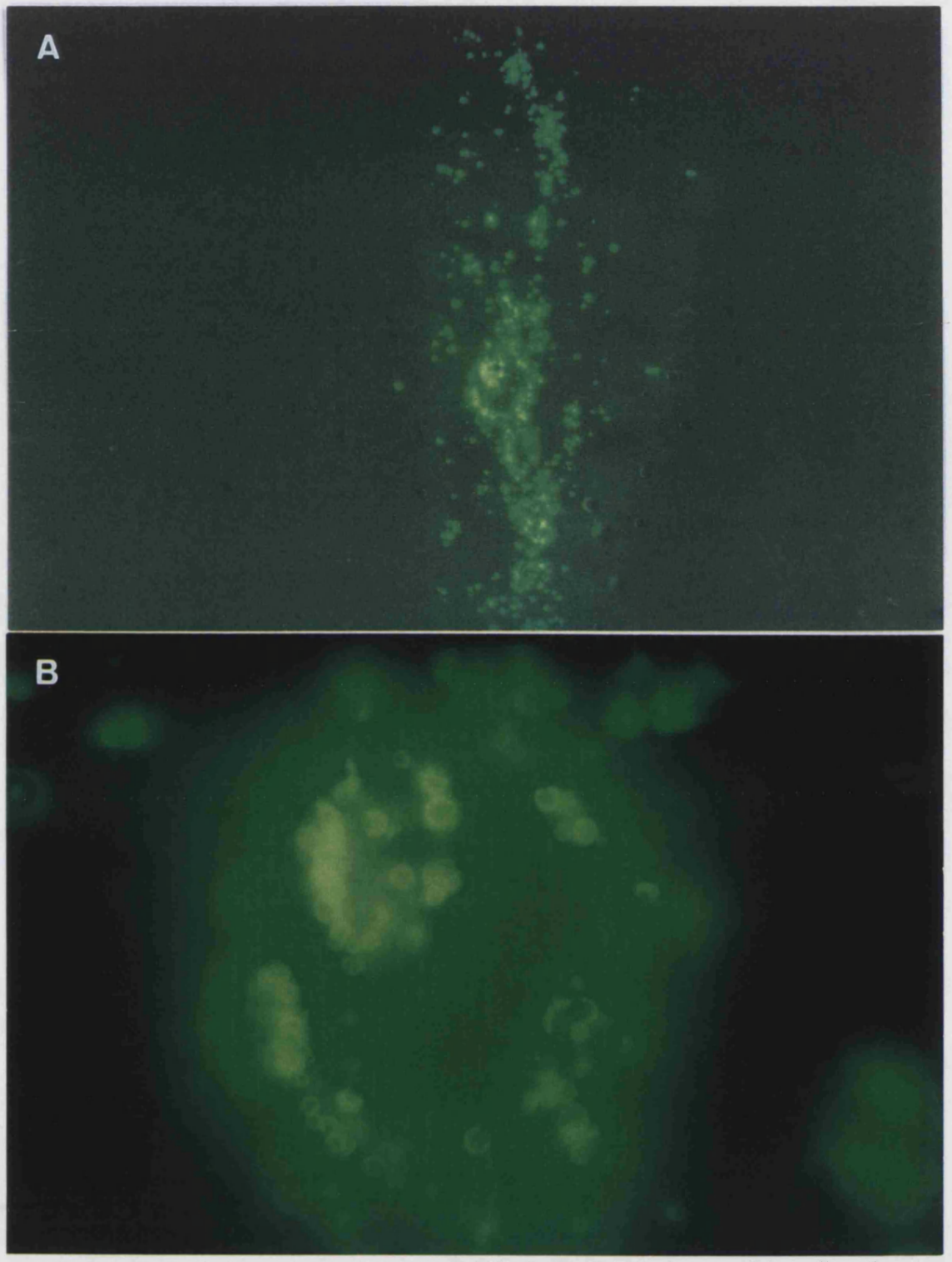


Figure 5.19. Typical fluorescent images of FITC labelled microparticles directly injected into solid tumours. Samples of 1.5 mg of FITC labelled microparticles were directly injected into solid tumours. The tumours were excised 2 days post injection. Image A was taken at x100 magnification and image B was taken at x600 magnification.

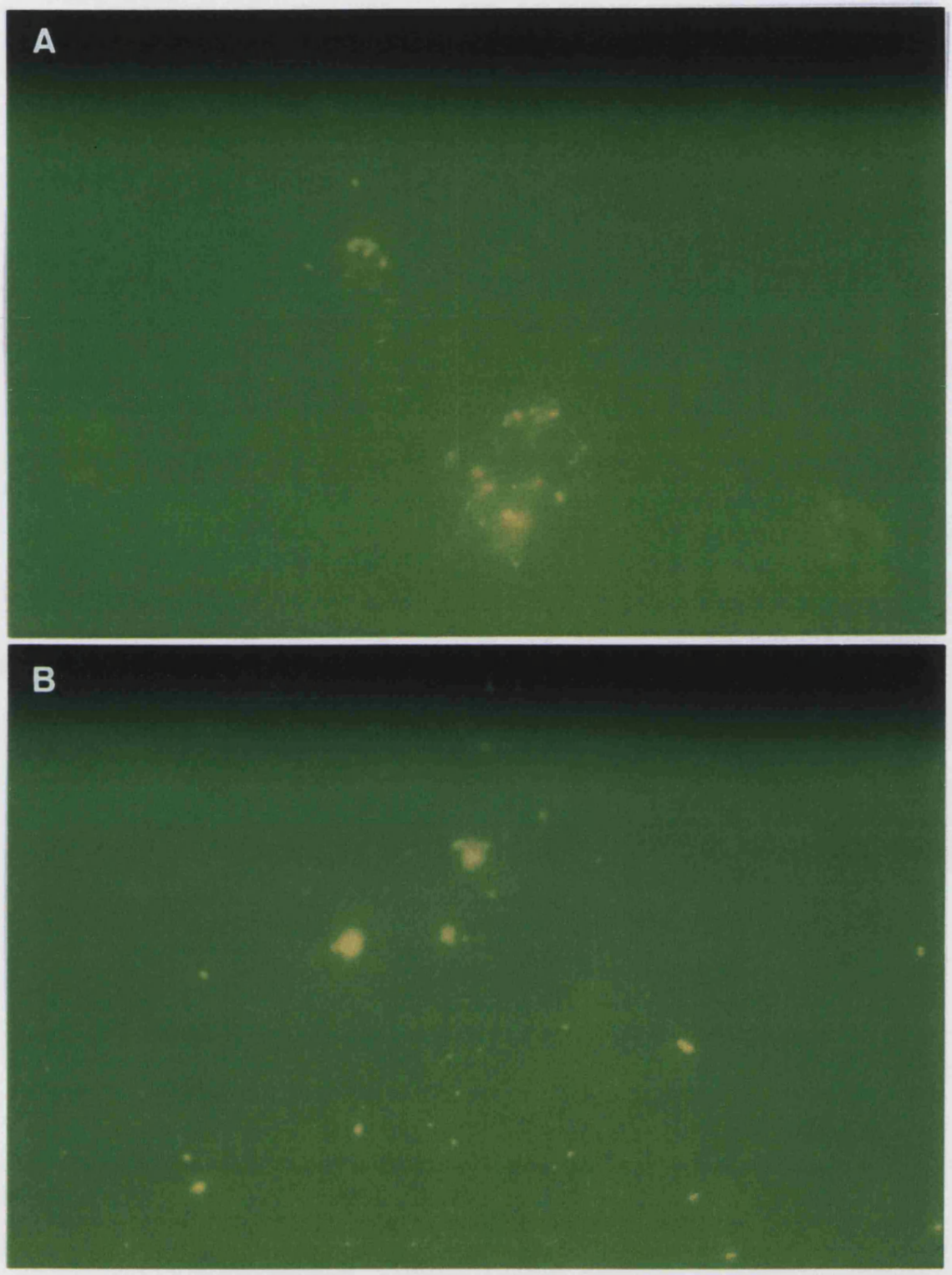


Figure 5.20. Typical fluorescent images of FITC labelled microparticles directly injected into solid tumours. Samples of 1.5 mg of FITC labelled microparticles were directly injected into solid tumours. The tumours were excised 7 days post injection. Images A and B were taken at x600 magnification.

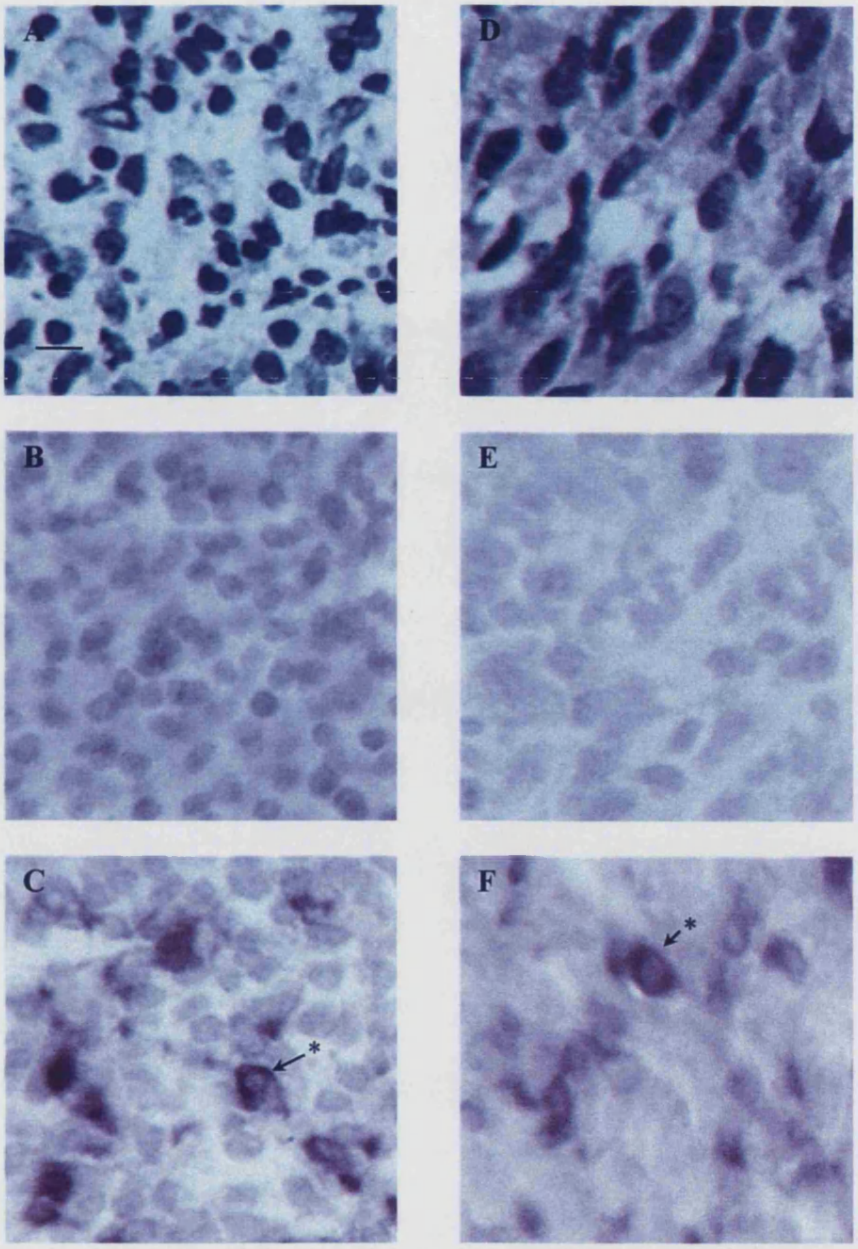


Figure 5.21. Enzyme histochemical staining of spleen (A-C) and tumour (D-F) tissue. Spleen (C) and tumour (F) tissue was treated with non-specific esterase (NSE). A & D are H&E stains of spleen (A) and tumour (D) to reveal nuclear structure of tissue. B&E are spleen (B) and tumour (E) negative controls for NSE staining. * denotes macrophages. Scale bar = 100 μm.

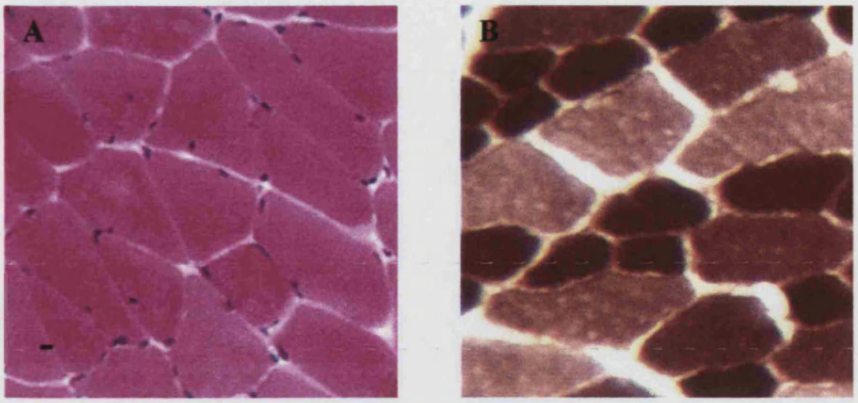


Figure 5.22. Enzyme histochemical staining of mouse muscle. A is an H&E stain of muscle tissue. B is the muscle tissue stained with non-specific esterase. Scale bar = 100μm.

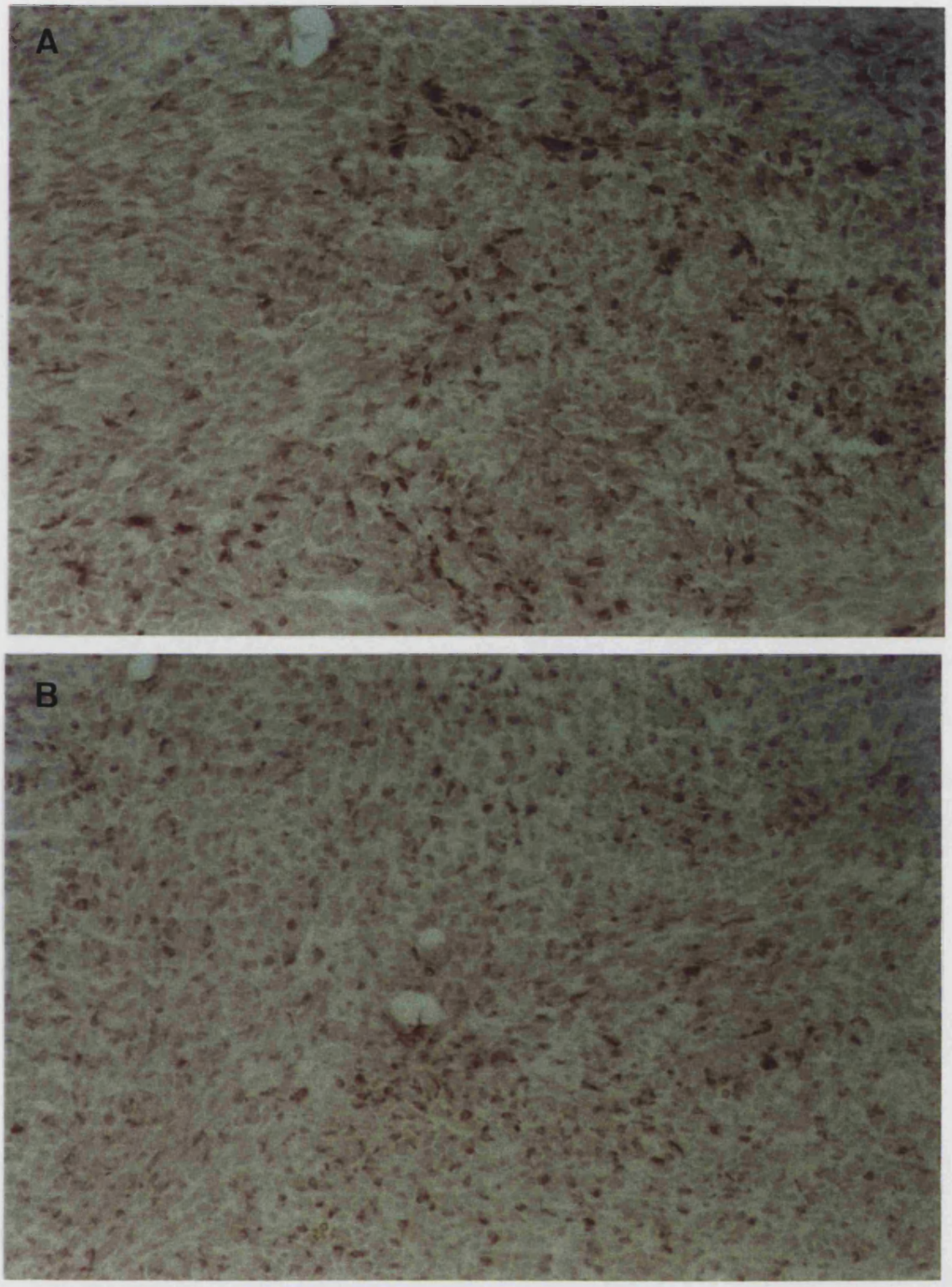


Figure 5.23. Images A&B show the distribution of macrophages in RIF-1 tumour tissue through non-specific esterase staining. Images were taken at x160 magnification.

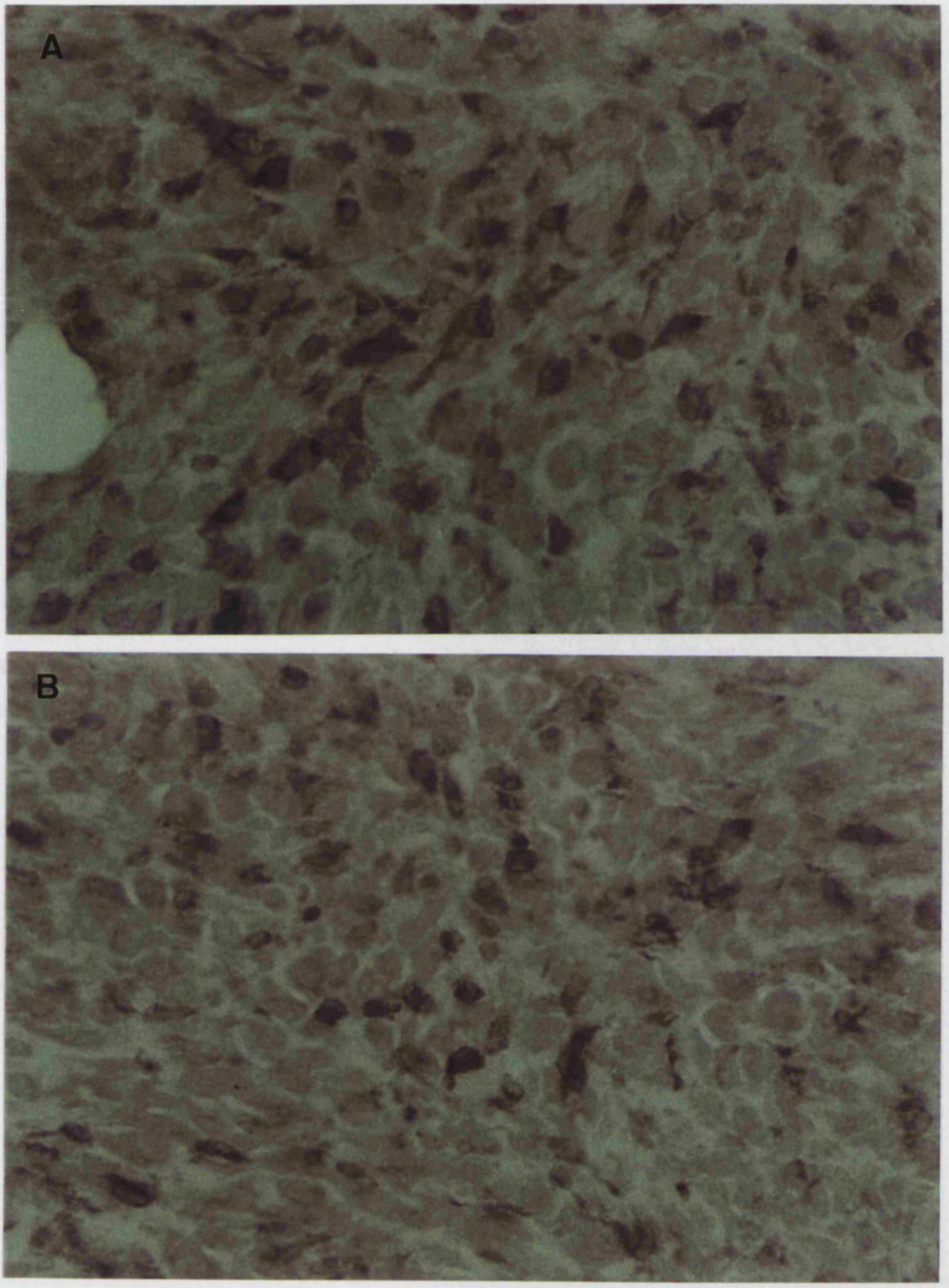


Figure 5.24. Images A&B show the distribution of macrophages in RIF-1 tumour tissue through non-specific esterase staining. Images were taken at x400 magnification.

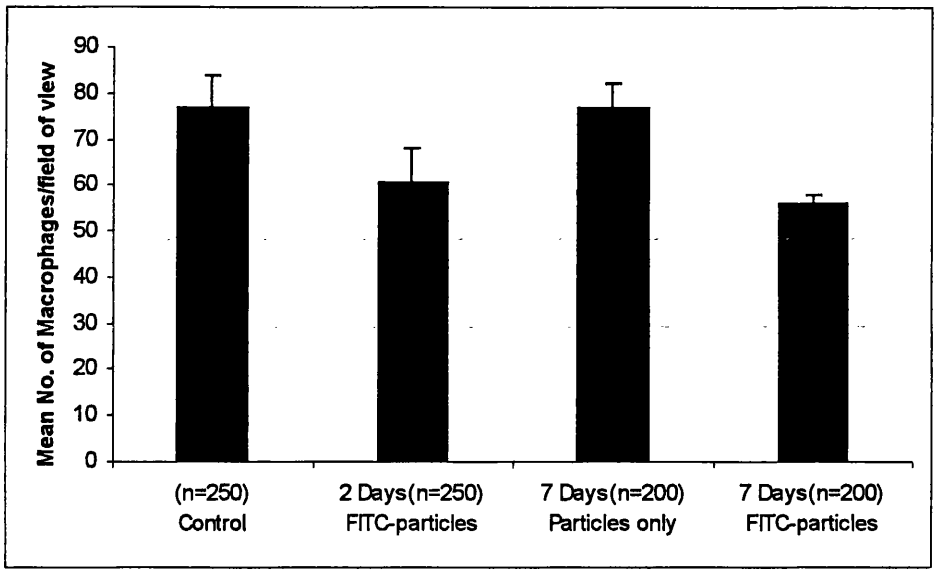


Figure 5.25. Effect of DHSA microparticles on the macrophage content of tumour tissue. 1.5 mg samples of both FITC and non-FITC labelled particles were directly injected into tumours. Control animals were not injected with microparticles. Field of view per treatment group n=250 or n=200. Data represents the mean of 250 or 200 (where applicable) samples ±SEM. For both 1 and 2 tailed t-test at P=0.05 it was found that the results of control group compared to the 2 day FITC-particles group (with 8 degrees of freedom), and control group compared with 7 day particles only (with 7 degrees of freedom), and control group compared with 7 day FITC-particles (with 7 degrees of freedom), and finally 7 day particles only compared with 7 day FITC-particles were all significantly different, see *Appendix E.*

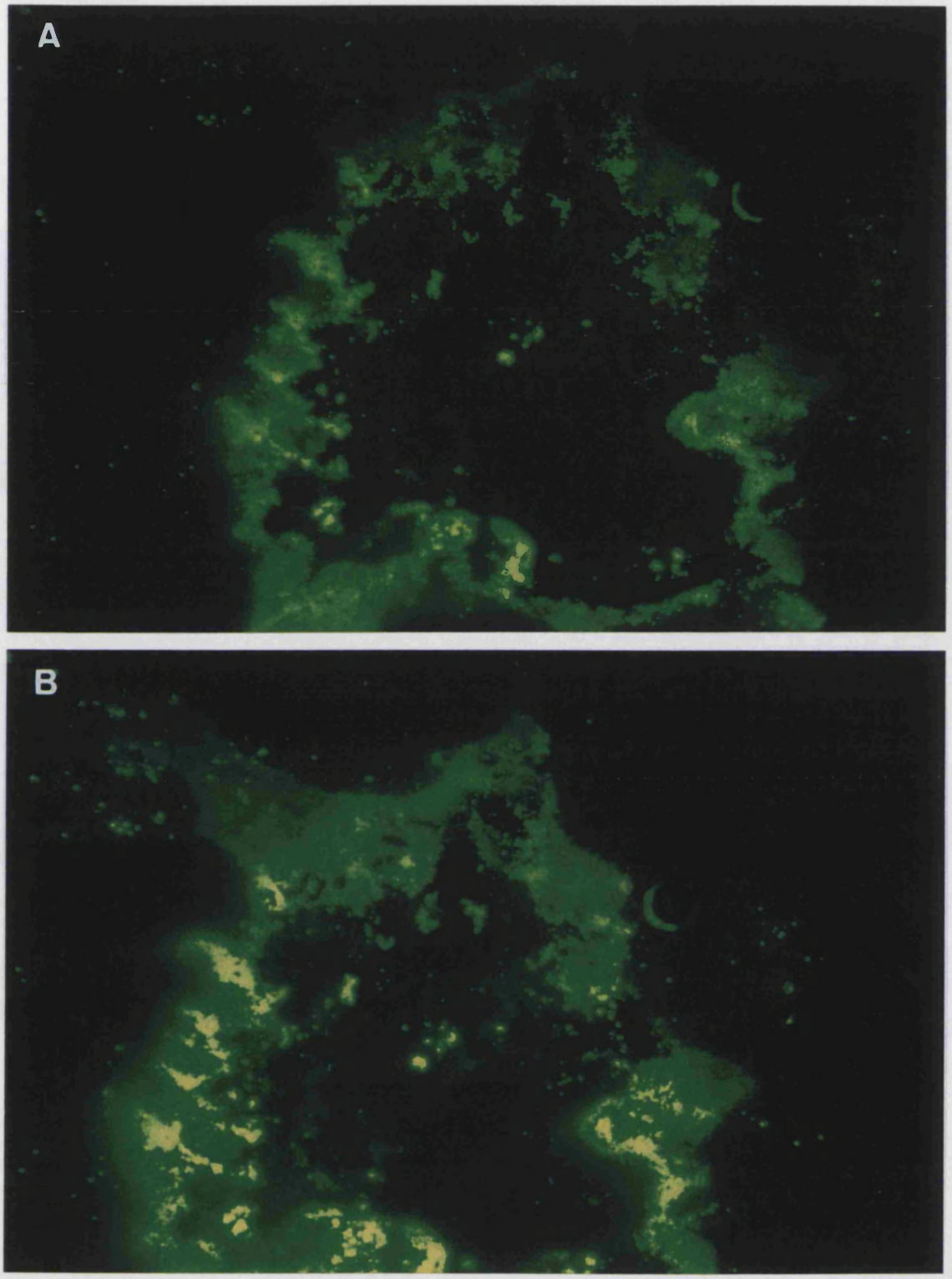


Figure 5.26. Typical fluorescent images of FITC labelled microparticles directly injected into solid tumours. Samples of 1.5 mg of FITC labelled microparticles were directly injected into solid tumours. The tumours were excised 2 hours post injection. Images A and B were taken at x100 magnification.

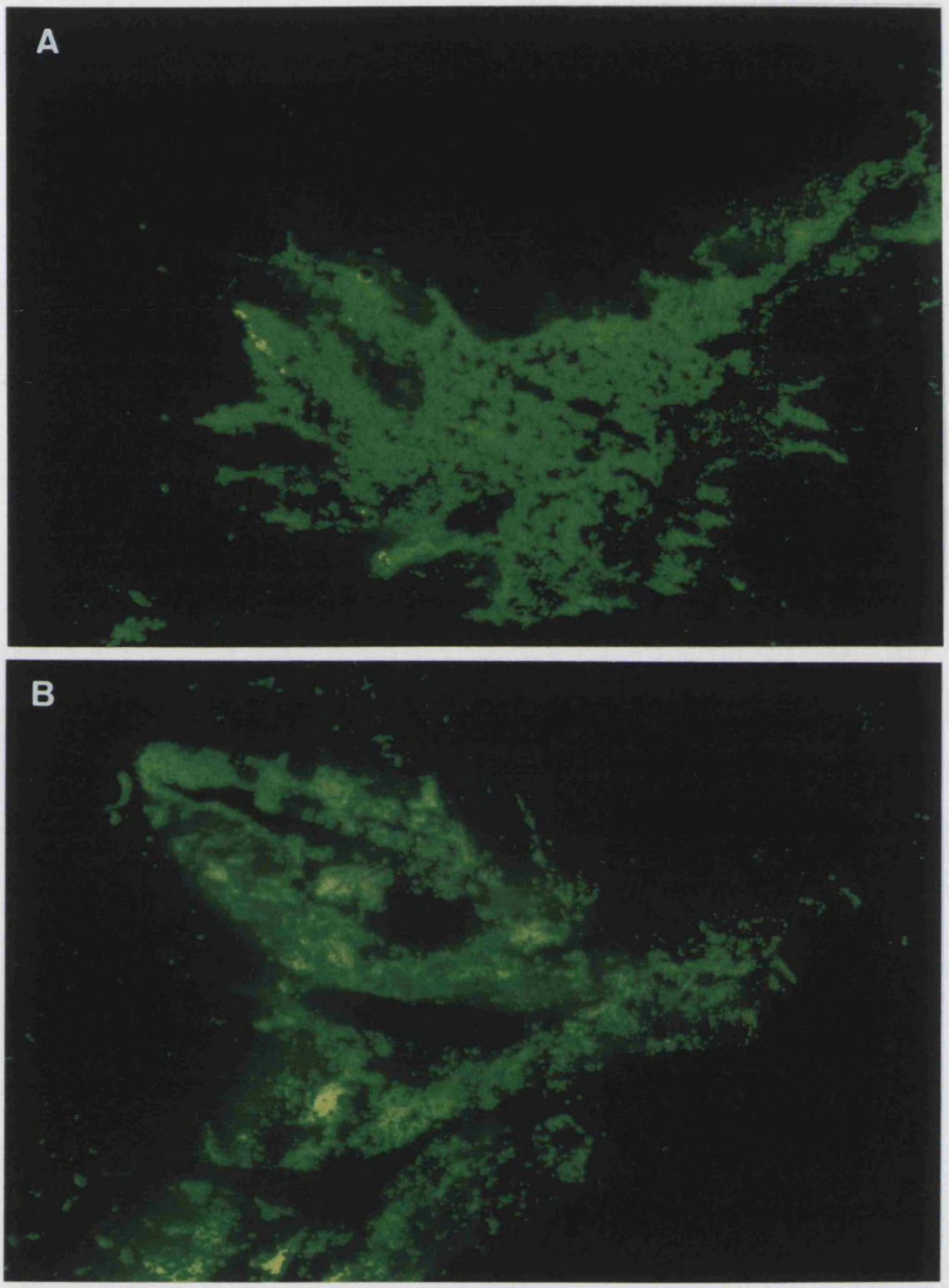


Figure 5.27. Typical fluorescent images of FITC labelled microparticles directly injected into solid tumours. Samples of 1.5 mg of FITC labelled microparticles were directly injected into solid tumours. The tumours were excised 24 hours post injection. Images A & B were taken at x100 magnification.

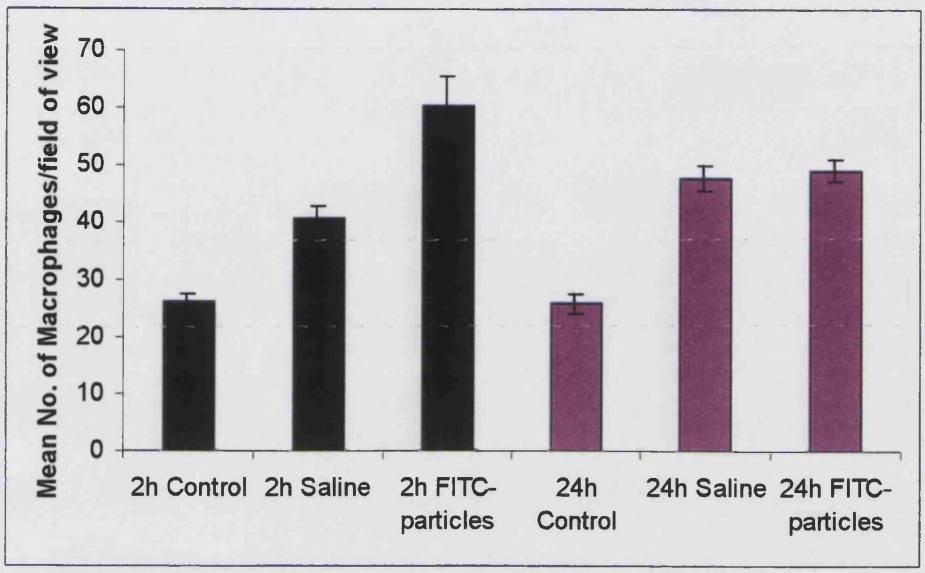


Figure 5.28. Effect of DHSA microparticles on the macrophage content of tumour tissue after 2 and 24 hour incubation post injection. Samples of 1.5 mg of FITC labelled microparticles were directly injected into tumour. Control animals were not injected with microparticles. Additional control animals were injected with saline. Field of view per treatment group n=250. Data represents the mean of 250 samples ±SEM. For both 1 and 2 tailed t-test at P=0.05 (given that there were 8 degrees of freedom), it was found that the results of 2h control compared to 2h saline; 2h saline compared to 2h FITC-particles; and finally 2h control compared to 2h FITC-particles were all significantly different. The results of 24h control compared to 24h saline; 24h saline compared to 24h FITC-particles; and finally 24h control compared to 24h FITC-particles were all significantly different, see *Appendix E.*

5.4. DISCUSSION

Among a variety of cancer gene therapy treatments currently in use, immunotherapy is the most promising. Generally the present strategies in immunotherapy involve the manipulation of the antigen processing and presentation pathway.

Antigen processing involves the degradation of externally or internally derived antigen into short peptide sequences and the association of the peptide with MHC molecules, either MHC class I or class II on antigen presenting cells and presentation of these peptide molecules on the surface of these antigen presenting cells. The location of the antigen, extracellular or intracellular, determines which MHC molecule will bind the peptide. Peptides derived from antigenic proteins that reside within the cell (or self produced) such as viral proteins, bind to MHC class I molecules. While peptides derived from antigenic proteins that reside outside of the cell (or non-self produced), such as either bacterial proteins or large proteins bind to MHC class II molecules. MHC class I molecules are found on the surface of all nucleated cells, however MHC class II are far more restricted in their distribution. B cells, macrophages, dendritic cells and in the human, activated T cells are among the most strongly positive for class II. An improved understanding of the molecular mechanism of antigen processing and presentation, and the identification of tumour-associated antigens (TAA) in melanoma and other cancers, have allowed the development of specific vaccines.

Peptide vaccines, consist of peptide sequences recognised by cytotoxic T lymphocytes (CTL), can induce CTL responses (Schulz *et al.*, 1991). These peptide vaccines can be formulated with immunostimulating complex, entrapped in liposomes, or coated on their surface. Peptide vaccines are advantageous as they can be designed to induce well-defined immune responses and can be synthesised with very high purity

and reproducibility in large quantities. They can also identify the specific epitopes of the tumour antigens to which an individual is able to mount an immune response. (reviewed by Minev *et al.*, 1999).

Another vaccine approach is the use of dendritic cells (DC) for inducing antitumour immune responses. DCs are the most potent APCs for the initiation of antigen-specific immune responses. In addition to their ability to efficiently acquire and process antigens, DCs express high levels of MHC Class I and Class II molecules, as well as co-stimulatory molecules essential in antigen presentation. Therefore many investigators have attempted to immunise with peptide-pulsed DCs. The results showed immunisation with peptide-pulsed DCs is superior to injection of peptide in adjuvant in inducing potent cytotoxic T-cell responses (reviewed by Minev *et al.*, 1999).

The use of heat shock protein (HSP)-peptide complexes for vaccination is also being investigated. Vaccination of mice and rats with HSP-peptide complexes has resulted in powerful immune responses against the peptide bound to HSP, but not the HSP itself (Udono *et al.*, 1994). A disadvantage of this approach is the requirement for generation of customised patient-specific vaccines for cancer.

DNA vaccines involve direct inoculation of expression plasmids, which result in the induction of long lasting immune responses against the expressed antigens. Several elegantly designed studies addressed the important question of the mechanism of DNA immunisation (Corr *et al.*, 1996; Ulmer *et al.*, 1996; Condon *et al.*, 1996). Results demonstrate that the APCs can be transfected directly or they can acquire antigens expressed by other transfected cells. However, only professional APCs are able to initiate primary immune responses as result of DNA immunisation. A promising DNA vaccine has been developed against a B-cell lymphoma (Syrengelas *et al.*, 1996). DNA vaccines have several potential advantages over peptide vaccines. DNA vaccines are

simpler and cheaper to produce, are not rapidly cleared from the host, and produce very long-lasting immune responses. In all DNA-based vaccines seem to be promising approach for the treatment of cancer.

The disadvantage of peptide-based vaccines is their low immunogenicity, therefore careful selection of the adjuvants used with these vaccines is important. The function of each adjuvant depends on its ability to affect the pathway of the antigen presentation to prolong the antigen exposure to the APCs, as well as to influence the number and the type of APCs, and the release of cytokines in the local environment. One promising new adjuvant that is currently being investigated is unmethylated CpG dinucleotides, found in bacterial DNA. CpGs cause direct immunostimulatory effects in the immune cells *in vitro*. They trigger cells of the innate immune system, including macrophages and DCs, to upregulate MHC class II and costimulatory molecules. They also bring about the conversion of immature DCs into mature APCs, however CpGs have mainly been evaluated in rodent models and with murine cells, thus their potency and safety in humans remains to be established (reviewed by Singh and O'Hagan, 1999).

The use of particulate adjuvants, an alternative to immunostimulatory adjuvants has been evaluated by several groups. Particulate adjuvants e.g. microparticles have comparable dimensions to the pathogens that the immune system evolved to combat, therefore particulate adjuvants are naturally targeted for uptake by APCs to facilitate the induction of potent immune responses. Immunostimulatory adjuvants may also be included to enhance the level of immune activation, or to focus the response through a desired pathway (e.g. Th1 or Th2) (reviewed by Singh and O'Hagan, 1999). The use of microparticles to target antigens to APCs is one approach. Primary candidates have been biodegradable and biocompatible polyesters or poly(lactide-co-glycolides) (PLGs) for the development of microparticles as adjuvants, as they have been used in humans

for many years as suture material and as controlled-release delivery systems (Okada and Toguichi, 1995). Microparticles appear to mediate their effects largely as a consequence of their uptake into DCs, macrophages and local lymph nodes. A particularly attractive feature of microparticles is their ability to control the rate of release of entrapped antigens (O'Hagan, 1998) as controlled release of antigens may allow the development of single dose vaccines.

This chapter has investigated whether the DHSA microparticles induced a humoral immune response *in vivo*, without the presence of therapeutic DNA. FITC labelled DHSA microparticles were assessed *in vivo* to investigate the relationship between the presence of the microparticles and the number of macrophages present in the tumour or muscle tissue. However, before the latter could be carried out, *in vitro* experiments were carried out to determine whether macrophages were able to phagocytose the microparticles and if so the time frame it would take for the macrophages to phagocytose the microparticles. The distribution of the microparticles in the tissue after injection and the length of time the particles resided in the tissue before degradation was also investigated.

Mouse peritoneal macrophages were collected by injecting warmed Hank's balanced salt solution (37°C), as described in section 5.2.2. (Mann, 1973). The salt solution was warmed to avoid activating the macrophages before collection, this is to ensure that if the macrophages become phagocytic it is due to the properties of the microparticles (Fernandez-Repollet and Swartz, 1988). Three general types of phagocytosis have been described: spontaneous, normal and immune. Spontaneous phagocytosis is serum-independent ingestion, relatively few phagocytes participate in spontaneous phagocytosis and few particles are ingested. However, the rate of phagocytosis can be enhanced, by increasing (1) the number of collisions between

phagocytes or particles, (2) the size and density of phagocytes and (3) the absolute number of particles or phagocytes. Normal phagocytosis is mediated by the normal serum opsonins or natural antibody, so called as the addition of normal serum enhances phagocytosis. Such non-specific opsonizing activity appears to reside in the alpha-2 globulin fraction. Phagocytic activity of phagocytes is dramatically enhanced by specific-immune antibody and further accelerated by complement. Specific opsonization, implies binding of the particle surface to specific antibody, induced by previous exposure to the antigen and directed against antigenic determinants on the particle surface (reviewed by Fernandez-Repollet & Swartz, 1988).

FITC-labelled microparticles were incubated with the mouse peritoneal macrophages for 2, 24, 48, and 72 hour periods and observed using microscopy. It appears that the microparticles did undergo the three phases of phagocytosis, the initial attachment, the ingestion and the digestion. The initial attachment and ingestion occurred as early as 2 hours and continued between 2-24 hours. The digestion process took place 24-72 hours *in vitro* conditions. This indicated that there were an adequate number of collisions between the phagocytes and the microparticles. The microparticles were generally within a similar size range as the macrophages. Less aggregated microparticles were phagocytosed more efficiently as expected due to their more manageable size. It can be assumed that the mechanism of uptake was spontaneous phagocytosis. However, as the microparticles were essentially spray dried human serum albumin and there was serum in the incubation medium (RPMI), the phagocytosis could also be regarded as normal phagocytosis.

Once the time-frame for phagocytosis had been established (note that macrophages were possibly activated by the presence of the FITC labelled microparticles), the next stage was to monitor the behaviour of the microparticles *in vivo*. Upon directly

injecting the FITC-labelled and unlabelled microparticles into tumour and muscle tissue, the distribution and stability of the microparticles in tumour tissue was observed using fluorescent microscopy. It was found generally, the majority of the microparticles were located in the periphery of the tumour tissue removed from the mid-region of the RIF-1 tumour near the site of injection (i.e the tumour can be divided into three areas top, middle and bottom). The particles appeared to be quite aggregated, possibly due to the mass of tumour tissue being quite tightly packed not to mention the intratumoral pressure. After 2 days *in vivo* the microparticles are still intact, however after 7 days the microparticles appear to have degraded (Figure 5.20). The degradation was most likely due to naturally occurring proteases gradually breaking down the albumin microparticle matrix. This localisation of microparticles and their long residence offers promise of controlled gene delivery of cancer vaccines.

Attempts were made to assess the adjuvant effects of these microparticles i.e. to determine if upon injection into tumour tissue they caused an immune response. To assess the immune response qualitatively and quantitatively macrophages were located within the tissue and cell counts were carried out. Initial attempts to locate macrophages and distinguish between residing and infiltrating macrophages, involved the use of immunocytochemical staining in particular the 'Avidin-Biotin' technique, which relies on the marked affinity of the glycoprotein for biotin. Essentially, a mono/polyclonal primary antibody was directed towards a specific determinant on the cells i.e. in this case rat-antimouse Mac-1(CD11b) for the general detection of macrophages. After the 1° antibody was applied biotinylated anti-rat IgG is added, followed by preformed complex between avidin and biotinylated enzyme (with enzyme substrate-alkaline phosphatase). This preformed complex consisted of many biotinylated enzyme molecules cross-linked by avidin into a three-dimensional array.

The complex apparently has few exposed biotin residues but has at least one remaining biotin site to bind to the biotin on the 2° antibody (Vector Laboratories, UK). The latter method did not work for the RIF-1 tumour as the 1° antibody did not appear to pick out individual macrophages but coated the whole of the tissue sections, indicating that a protein on the surface of the tumour cells was reacting with the Mac-1. Leek *et al*, have found in invasive breast carcinomas, that the neoplastic cell population is often out numbered by such stromal cells as tumour-associated macrophages, which can comprise more than 50% of the total tumour mass. (Leek *et al.*, 1996, 1998). The latter observation could also offer a possible explanation on why the Mac-1 general macrophage marker coated the majority of the RIF-1 tumour tissue sections, as the use of the RIF-1 tumour model up until now has only been associated with *in vivo* evaluation of chemotherapy and radiotherapy (Twentyman *et al.*, 1980). As it was clearly difficult to detect macrophages using Mac-1, attempts to detect dendritic cells using CD11c were also futile, as a relationship between the quantity of macrophages and dendritic cells could not be observed using immunocytochemical staining.

An alternative method for the detection of macrophages was sought out, enzyme histochemistry is one alternative method. Enzymes are vital components of biological systems, they may be free and soluble in the cytoplasm or body fluids (lysoenzymes) or bound to specific cell components (desmoenzymes). In the case for detecting macrophages esterase enzymes were of interest as these hydrolytic enzymes are found in the macrophages (Fernandez-Repollet & Swartz, 1988). The majority of esterase enzymes are able to hydrolyse α naphthyl acetate as a substrate, these enzymes are called 'non-specific' esterases. The enzymes release α naphthol during the hydrolysis of the substrate. The α naphthol is then coupled with a suitable diazonium salt, to produce an insoluble azo dye at the site of enzyme activity. The diazonium salt,

hexazotized pararosaniline was used as it gives good localisation of the enzyme (Bancroft & Stevens, 1996).

Once the macrophages had been identified, macrophage counts were carried out. The results from the first *in vivo* experiment, where RIF-1 tumours were treated with FITC-labelled microparticles and excised after 2 and 7 day periods, (Figure 5.25) appear to show a decrease in the number of macrophages in the tumours treated with FITC labelled microparticles. However, there seems to be no decrease in the number of macrophages present in tumours treated with unlabelled microparticles. The latter phenomenon could be attributed to the presence of FITC.

A second *in vivo* experiment was performed, this time tumours were excised 2 and 24 hours post injection (Figure 5.28). Injecting tumours with saline caused an increase in the number of macrophages found in tumour tissue. There was a further increase in the number of macrophages in tumour tissue, upon injection of FITC-labelled microparticles. However in tumours excised 24 hours post injection there seems to be a decrease in the number of macrophages present in tumours injected with FITC-labelled microparticles. The latter phenomenon could be explained by infiltrating macrophages migrating away from the tumour.

It is clear that the microparticles are taken up by peritoneal macrophages *in vitro*. An immune response is also mediated by the presence of the microparticles *in vivo*. However, more clinical work needs to be carried to establish whether the microparticles are taken up by the immune system and taken to the local lymph nodes, before DNA loaded microparticles can be investigated *in vivo*.

CHAPTER 6

CONCLUDING REMARKS

Gene therapy has enormous promise as a strategy for providing safe and effective therapies for some disorders. To fulfil this promise, approaches to gene therapy must be developed that are consistently effective and are also as safe and cost effective as conventional pharmaceutical and biotechnology products. These are familiar clinical challenges in drug development challenges against which methods for gene therapy and progress towards developing gene therapy products must be measured.

Biodegradable microparticles are already a well-established form of drug delivery, currently used for sustained delivery of hormones and vaccines to mention a few (Cleland, 1997). Currently their potential for use in the field of gene delivery is being explored (Schaefer & Singh, 1997, O'Hagan, 1997).

The objective of the research described in this thesis was to investigate the potential of spray-dried microparticles produced by an established spray-drying method at Quadrant Healthcare, for use in the field of gene delivery to the lung and solid tumours.

Spray drying is an extremely useful technique for producing powders to predetermined specifications, which was shown in Chapter 2 when spray drying mannitol microparticles. Spherical microparticles of a narrow size distribution between 3-6 μm were produced. The DNA retained its biological activity, which was determined via *in vitro* transfection of mammalian cells, with the re-dispersed lipoplexes. Further investigation is required however, as the spray drying of lipoplexes still needs a lot of work. The product recovery was low, which is expected with laboratory scale spray dryers (Broadhead, 1994) but as DNA is very costly regarding

time and money to obtain, further work needs to be done to optimise the conditions further to avoid loss of the DNA, but keeping in mind the heat sensitivity of DNA. Other areas that need further investigation are the moisture content of the mannitol particles as it has been reported not to be the most stabilising excipient to use when spray drying a biologically active compound (Broadhead *et al*, 1994), therefore alternatives need to be investigated. The *in vitro* transfection experiment was adequate to determine if the DNA had retained its biological activity after undergoing spray drying but the next step would be to take this *in vivo* as with all drug deliveries their behaviour *in vitro* does not reflect their behaviour *in vivo*. Therefore a suitable *in vivo* lung model would have to be sought to evaluate clinical response, mode of deposition, the effect of mucocillary clearance and most importantly the safety of potential carriers. The other key area of investigation would be an alternative gene delivery vector to DOTAP, which has been recently reported to be inefficient when transfecting *in vivo*.

The potential for using microparticles for DNA vaccination strategy by introducing plasmid DNA encoding antigenic proteins via intratumoral delivery into host cells was investigated in Chapter 4. As with the mannitol formulation the specifications adopted for spray drying the DHSA suspension also gave poor product recovery reiterating the need for process optimisation. DNA was adsorbed onto the surface of DHSA microparticles with the aid of polycations. However, further experiments need to be carried out to determine a more efficient way of adsorbing DNA onto the surface of these microparticles in order that these microparticles can provide a sufficient depot of therapeutic DNA. Another alternative may be to spray dry several different types of polycations with defatted human serum albumin at different percentages of the overall spray drying suspension to see if DNA can be adsorbed more efficiently when directly loaded onto the surface of these microparticles. Using YOYO-

1 to monitor the adsorption of DNA onto microparticles was adequate, however its sensitivity did make it difficult to monitor adsorption at saturation levels where the DNA adsorbed was close to the DNA remaining in solution. Therefore for future work it would be useful to explore the possibility of finding an alternative, such as a reporter gene that is pre-labelled with a chromophore which gives absorption at a pre-determined wavelength.

Once it was determined that it was possible to adsorb DNA onto the surface of these microparticles these DNA loaded microparticles were used to transfect *in vitro*. Very little gene expression was obtain, this is probably because B16 mouse melanoma is a monolayer and the microparticles were too big to be taken up for transfection. The little transfection obtained was probably due polyplexes that had been shed from the microparticles. Further transfection experiments need to be carried out with active cells such as peritoneal macrophages or a macrophage cell line as the macrophages are able to engulf these microparticles and digest them, which was shown in chapter 5. Peritoneal macrophages were shown to engulf microparticles *in vitro*, which was demonstrated with fluorescent images. The following *in vivo* experiments showed that the presence of these microparticles stimulated an immune response, but further experiments need to be carried to verify whether these microparticles are taken up by macrophages *in vivo*, this could be achieved by locating and analysing local lymph nodes. Work also need to go into establishing the stability of these microparticles *in vivo*. The RIF-1 tumour model was difficult to analyse in terms of identifying specific immune cells, as it is normally used for radiation studies, therefore for future work it would be wise to investigate a model which was established as a means of monitoring immunity in tumours *in vivo*. Overall, a lot of clinical work needs to be carried out to verify the potential of these microparticles for intratumoural vaccines.

The future work mentioned was not carried out due to time and money constraints as well as limited access to equipment. The results obtained showed great promise for using microparticles in the field of gene delivery. Although we still have a long way to go, as with conventional products a lot of expensive research over along period of time has to be carried to get closer to having a gene therapy product on the shelves.

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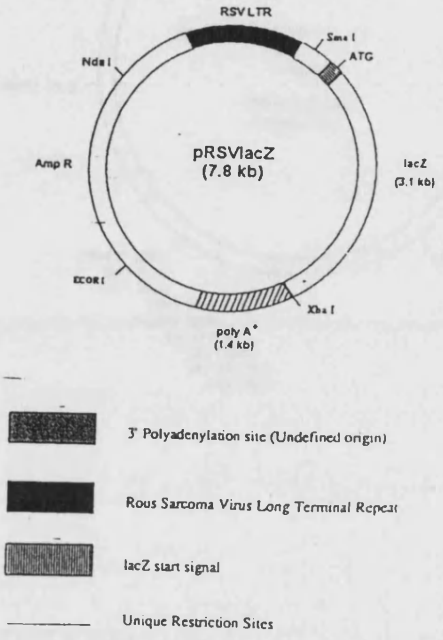
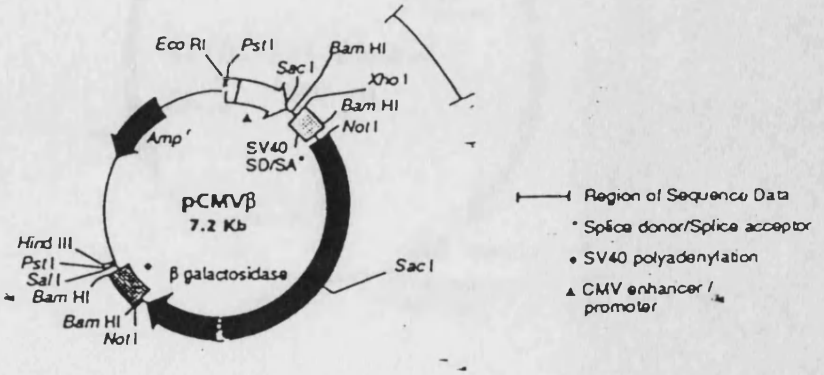
APPENDIX A

Plasmid Maps

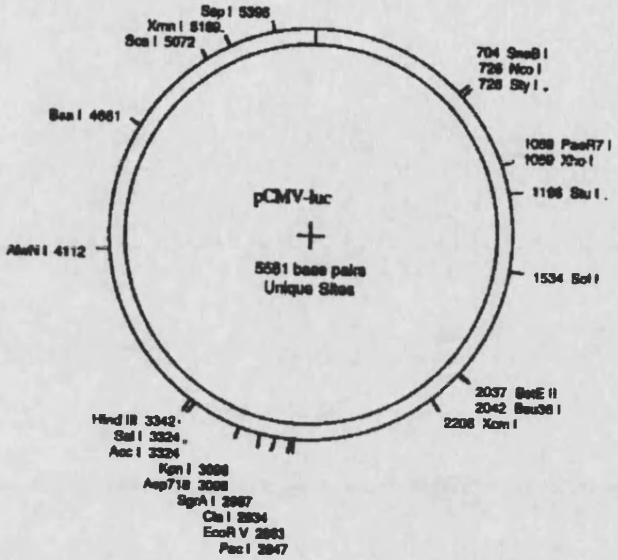
Restriction Map of pRSVlacZ



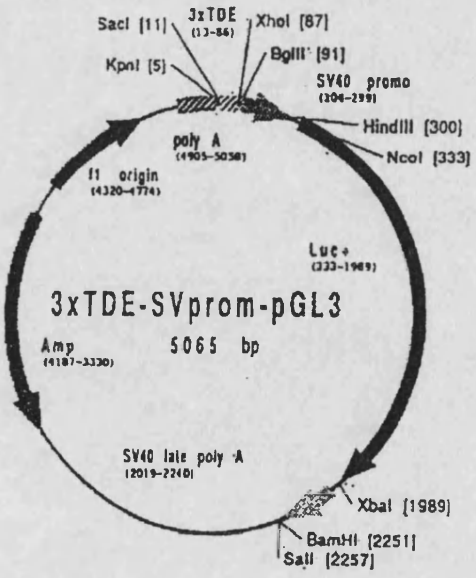
Restiction Map for pCMVlacZ



Restriction Map for pCMV*luc*



Restriction Map for pTDE SV40



APPENDIX B

Molecular Methods

B.1.Molecular Weight Marker

For agarose gel electrophoresis, the marker used was EcoR I/Hind III cut λ DNA (NBL Gene Sciences Ltd, UK).

1	**21226**	6	**2027**	11	**831**
2	**5148**	7	**1904**	12	**564**
3	**4973**	8	**1584**	13	**125**
4	**4268**	9	**1375**		
5	**3530**	10	**947**		

B.2. Antibiotics

1. Ampicillin sodium (Sigma, UK) was prepared as a 100 mg/ml solution in water and filter sterilised using a 0.2 μm pore membrane (Millipore, UK) and stored at -20 °C for upto 3 months.

2. Tetracycline (Sigma, UK) was prepared as 25 mg/ml solution in methanol and stored at –20 °C for upto 3 months.

B.3.Solutions

TAE buffer 0.04 mM Tris-Acetate
0.001 mM EDTA

TE buffer 10 mM Tris. Cl (pH 8)
1 mM EDTA (pH 8)

Electrophoresis Loading Buffer (5x)

0.25% Bromophenol blue
0.25% Xylene cyanol FF
15% Ficoll (type 400, Pharmacia)

Luria Bertani Medium (LB)

Bacto-tryptone		10 g
Bacto-yeast extract		5 g
Sodium Chloride		10 g
DDDW	to	1000 ml

LB Agar

Bacto-tryptone		10 g
Bacto-yeast extract		5 g
Sodium Chloride		10 g
Agar Technical		12 g
DDDW	to	1000 ml

Reagents for preparing LB were obtained from Difco, UK. Solutions were sterilised by autoclaving at 121 °C and 15 psi for 15 minutes on a liquid cycle in a Swingate Type SFT-LAB oven (British Steriliser Co. Ltd., UK)

B.4. Preparation of Competent E. coli DH5α cells

TfbI Buffer

30 mM KCL		3 ml of a 1 M solution
100 mM RbCl		1.209 g
10 mM CaCl2		12.5 ml of 80 mM solution
50 mM MnCl2		5 ml (1 M solution)
15% (v/v) Glycerol		15 ml
Water	to	100 ml

Adjust to pH 5.8 with 0.2 M acetic acid and filter sterilise.

TfbII Buffer

10 mM MOPS		0.209 g
75 mM CaCl2		1.1 g
10 mM RbCl		0.1209 g
15% (v/v) Glycerol		15 ml
Water	to	100 ml

Adjust to pH 6.6 with 1 M KOH and filter sterilise.

A single colony of E. coli DH5α was picked from a fresh agar plate and inoculated into 5 ml of LB medium and incubated at 37°C overnight in a shaking incubator. The following morning, 1 ml of the culture was diluted to 100 ml with LB and grown under standard conditions until the optical density (OD550nm) of the solution reached 0.48 (2.5-3 hours). The cell suspension was then chilled on ice for 5 minutes and centrifuged in sterile falcon tubes for 5 minutes, 6000 rpm (Beckman J2-MC) at 4°C. The supernatent was discarded and the cells re-suspended in 40 ml of TfbI buffer ($2/5^{th}$ volume of culture) and chilled on ice for 5 minutes. Cells were centrifuged at 6000 rpm for 5 minutes at 4°C and the supernatant discarded. Cells were subsequently re-suspended in 4 ml ($1/25^{th}$ volume of culture) of TfbII buffer and chilled on ice for 15 minutes. Cells were stored in 200 µl aliquots, snap frozen in a dry-ice/methanol bath and stored at -70°C until required.

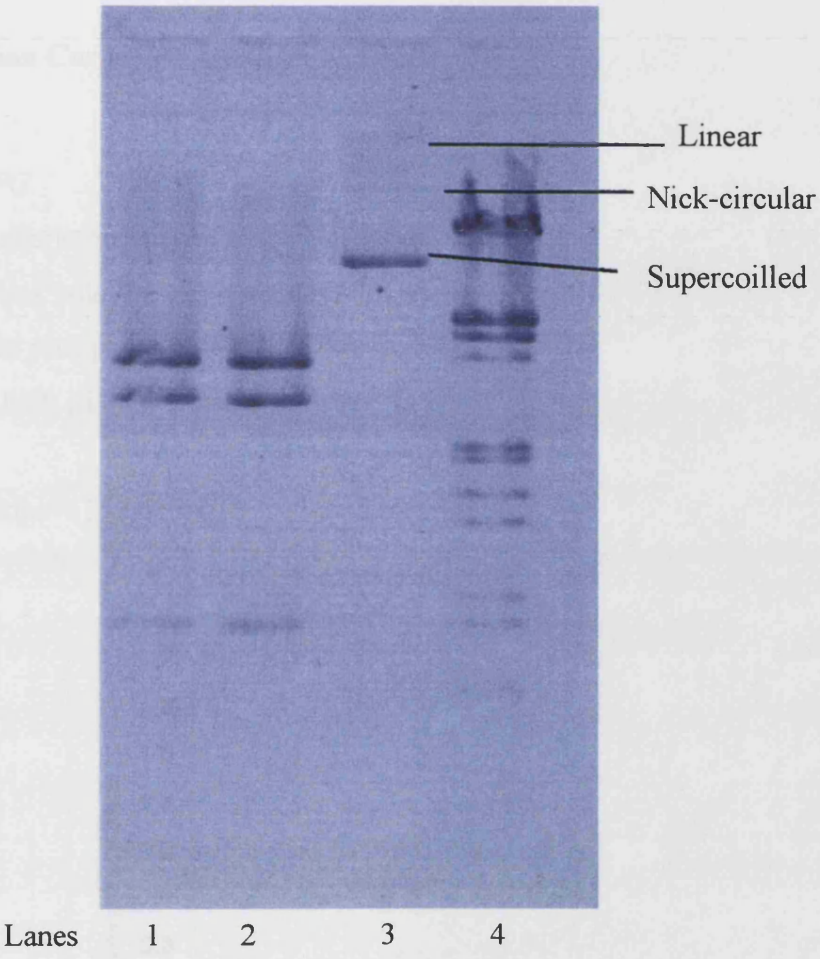
B.5. Transformation of Competent E. coli DH5α Cells

Two hundred microlitres of competent cell suspension was thawed at room temperature and then placed on ice for 10 minutes. Plasmid DNA (<100 ng) was then added and gently mixed using a Gilson tip followed by incubation on ice for 30 – 45 minutes. The cells were shocked by incubating at 37°C for 2 minutes and then returned to ice for 2 minutes. The cell suspension was then diluted to 800 µl with LB followed by incubation at 37°C for 1 hour. After this time, 100 µl of this suspension was plated out on a LB-agar plate containing ampicillin at a concentration of 100 µg/ml, or other antibiotic depending on the plasmid. Plates were incubated overnight at 37°C.

B.6. Colony Picking and Plasmid Purification

After checking the controls, sterile toothpicks (autoclaved) were used to transfer single colony forming units from the LB plates into 10 ml of LB broth containing ampicillin at a concentration of 100 µg/ml. The plasmid was then propagated, isolated, purified and quantified as described in chapter 2. The DNA sample was then screened using a restriction digest followed by gel electrophoresis and the undigested plasmid was loaded onto a gel and sized using a supercoiled ladder.

B.7. A 0.8% agarose gel showing the restricted sites on the pCMVlacZ plasmid from E.coli XL1 Blue.



Lane 4 DNA EcoR I/Hind III Digest

Lane 3 pCMVlacZ (unrestricted)

Lane 2 pCMVlacZ (Not 1, Pst 1 restriction, Not 1-3530 base pairs, Pst 1-2675 base pairs, Pst 1-Not 1 855 base pairs, Not 1-Pst 1 196 base pairs rarely seen.)

Lane 1 pCMVlacZ (pCMVlacZ (Not 1, Pst 1, EcoR I restriction, Not 1-3530bp, Pst 1-2675bp, Pst 1-Not 1 855bp, Not 1-Pst 1 196bp and EcoR I-Pst 1 are rarely seen.)

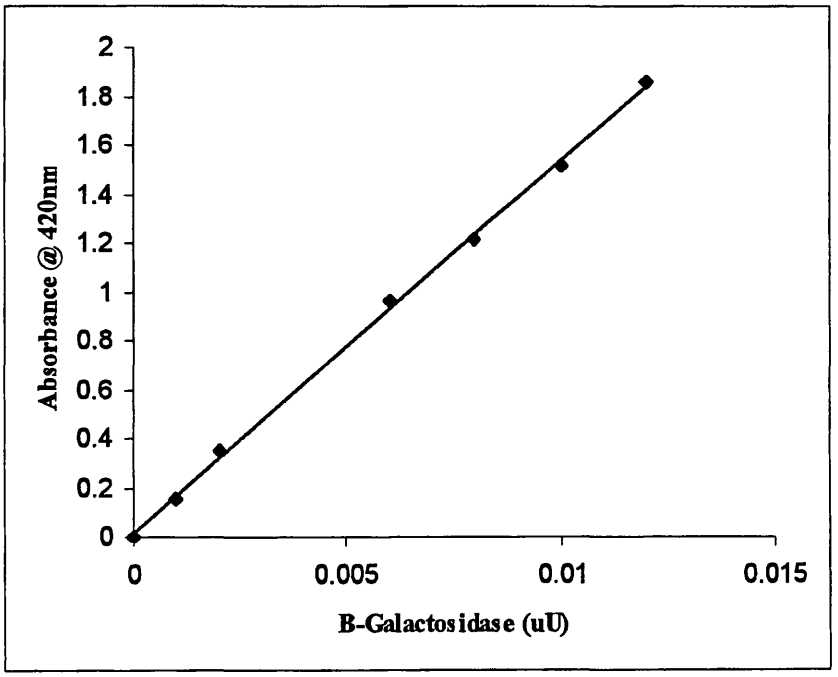
APPENDIX C

Calibration Curves for Quantitative Assays

C1. ONPG

Calibration curve for ß-galactosidase using ONPG as a standard. From a 0.2 mU/ml stock solution of enzyme (ONPG)(Sigma) 300 µl samples were prepared in 0.1 M Sodium phosphate buffer (pH 7.4) containing 0, 0.001, 0.002, 0.004, 0.006, 0.008, 0.010, 0.012 µU of ß-galactosidase. The following linear regression analysis was obtained:

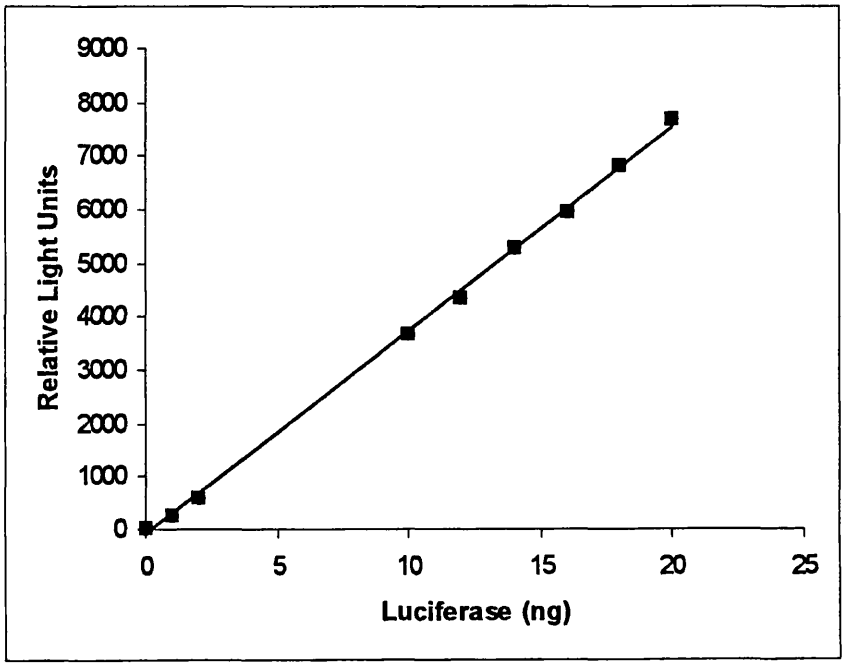
Slope = 152.67 μU^{-1} Intercept = 0.0156 R^2= 0.9987



C2. Luciferase assay

A series of solutions was prepared by dilution of purified luciferase (Promega) stock with 1x lysis buffer (Promega). Samples were produced which contained 0, 1, 50, 100, 500, 600, 700, 800, 900, 1000 ng of luciferase in 20 µl. Analysis was performed as described in 3.2.5.2. A Beer-Lambert plot for light units was determined with the following regression analysis:

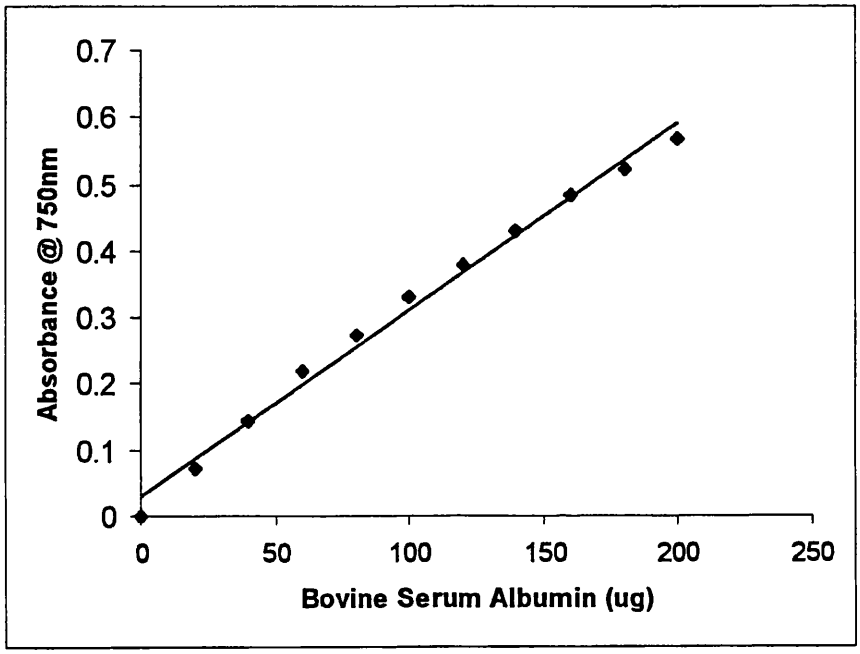
Slope = 381.78 ng^{-1} Intercept = -82.716 R^2= 0.9989



C3. Assay for soluble protein content of cell extracts

Calibration curve for protein using bovine serum albumin (BSA) as a standard and the Bio-Rad Dc protein assay kit. From a 2 mg/ml stock solution of BSA (Sigma) 100 µl samples were prepared in 0.1 M sodium phosphate buffer (pH 7.4) containing 0, 20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 µg of protein. The following linear regression analysis was obtained:

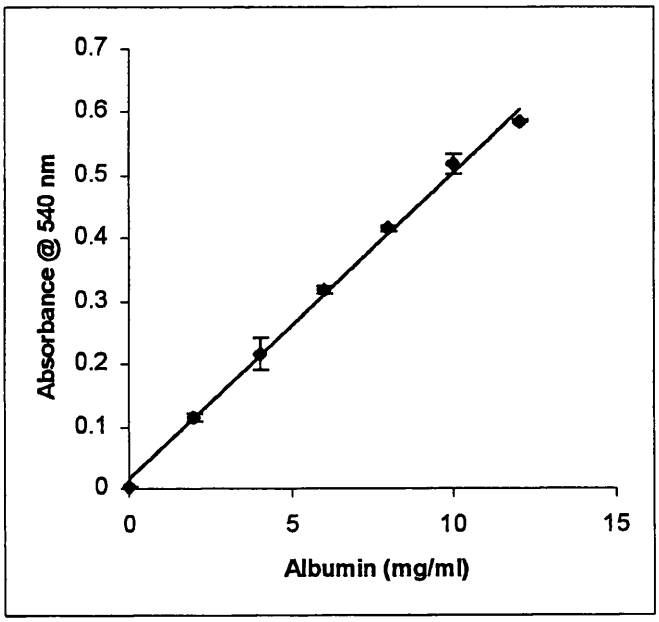
Slope = 0.0028 μg^{-1} Intercept = 0.0295 R^2= 0.9914



C4. Biuret Assay

Calibration curve for protein using albumin as a standard and the Biuret reagent (3 g of copper sulphate and 9 g of sodium potassium tartrate dissolved in 500 ml of 0.2 mol/L sodium hydroxide; added 5 g potassium iodide and made upto 1 litre with 0.2 mol/L sodium hydroxide). Samples were prepared containing 0, 2, 4, 6, 8, 10, 12 mg/ml of albumin. The following linear regression analysis was obtained:

Slope = 0.0493 ml mg^{-1} Intercept = 0.0144 R^2= 0.997



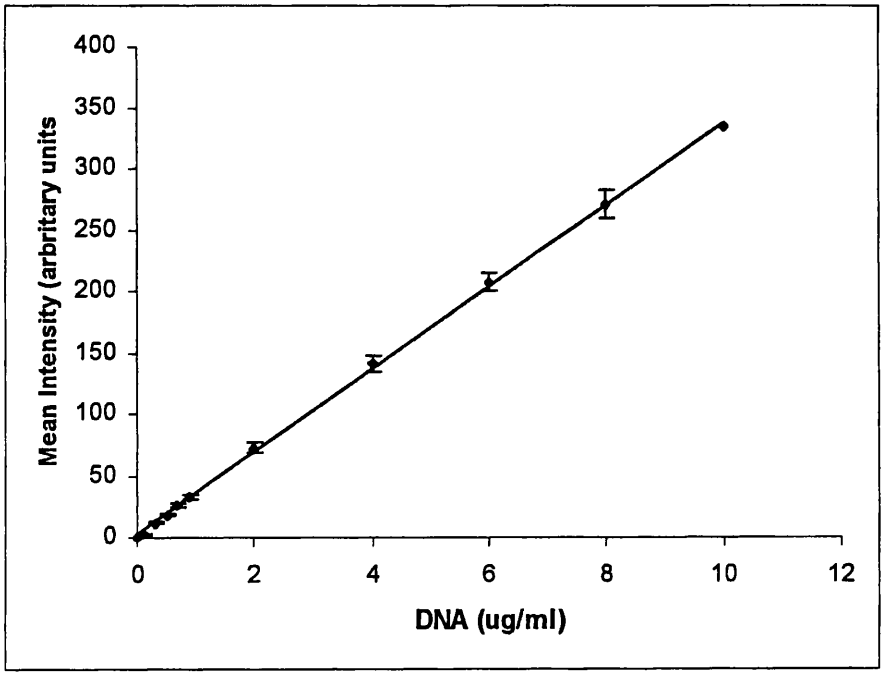
APPENDIX D

Nucleic Acid Staining

Taking into account that the molar ratio of 50 base pairs:1 dye was used in the calculation of the absorption and fluorescence maxima, 50 base pair: 1 ratio was taken as the starting point to obtain a calibration curve (RMM base pair = 660, YOYO-1 = 1271). Serial dilutions of stock solution of 50:1 base pair/dye ratio were made.

Aqueous solutions of dimeric cyanine dyes without added DMSO are unstable due to surface adsorption. To circumvent this problem aqueous solutions were prepared by diluting from the DMSO stock solution immediately before they were required for use, in plastic rather than glass containers and left to stabilise for 10 minutes before readings were taken at excitation 491 nm and emission 509 nm. Minimal transfer steps were adopted to further circumvent surface adsorption. The following linear regression analysis was obtained:

Slope = 33.584 ml μg^{-1} Intercept = 2.4917 R^2= 0.9995



APPENDIX E

Animal studies

All tumours collected were sectioned all the way through at 5 μm per section, and every 5 th section was qualitatively assessed via fluorescence microscopy. For macrophage counts, consecutive sections from the mid-region of the tumours were collected for enzyme histochemical staining and counts were made manually at x250 magnification, see tables a & b. The optimum number of sections per animal and number of fields per section were determined using 10 fields from each of 10 consecutive sections of RIF-1 tumour model. The cumulative number of fields that gave the lowest coefficient of variation was chosen as the number of fields per section to be quantified. The number of sections per tumour model was selected to give a SE ±25% of the mean.

Table a

Experiment 1 Treatment	Number of Animals	Number of consecutive sections per tumour	Field of view per section
Control (untreated)	5	5	10
2 days FITC microparticles	5	5	10
7 days non-FITC microparticles	4	5	10
7 days FITC microparticles	4	5	10

Table b

Experiment 2 Treatment	Number of Animals	Number of consecutive sections per tumour	Field of view per section
2 hour control (untreated)	5	5	10
2 hour saline	5	5	10
2 hour FITC microparticles	5	5	10
24 hour control (untreated)	5	5	10
24 hour saline	5	5	10
24 hour FITC microparticles	5	5	10

Statistical Analysis

The tables below show the results from both the one and two tailed t-test at P=0.05, for both animal experiments.

In vivo 1

		Control compared to 2 day FITC particles	Control compared to 7 day particles only	Control compared to 7 day FITC particles	7 day particles only compared to 7 day FITC-particles
Variable 1	Mean	76.768	76.768	76.768	76.69
	Variance	52.462	52.462	52.462	31.183
Variable 2	Mean	60.687	76.69	56.02	56.02
	Variance	54.734	31.183	4.583	4.583
Observations	Variable 1	5	5	5	4
	Variable 2	5	4	4	4
Degrees of freedom		8	7	7	6
P(T<=t) 1-tail		0.004203	0.493201	0.000467	0.000227
Critical t 1-tail		1.859548	1.894578	1.894578	1.943181
P(T<=t) 2-tail		0.008406	0.986402	0.000933	0.000453
Critical t 2-tail		2.306006	2.364623	2.364623	2.446914

In vivo 2

		2h Control compared to 2h Saline	2h Saline compared to 2h FITC particles	2h Control compared to 2h FITC particles	24h Control compared to 24h Saline	24h Saline compared to 24h FITC particles	24h Control compared to 24h FITC particles
Variable 1	Mean	26.208	40.808	26.208	25.88	47.74	25.88
	Variance	1.544	3.431	1.544	2.908	5.728	2.908
Variable 2	Mean	40.808	60.456	60.456	47.74	49.076	49.076
	Variance	3.431	24.464	24.464	5.728	3.609	3.609
Observations for variables 1 &2		5	5	5	5	5	5
Degrees of freedom		8	8	8	8	8	8
P(T<=t) 1-tail		2.33E-07	1.65E-05	1.91E-07	8.625E-08	0.178437	1.8E-08
Critical t 1-tail		1.859548	1.859548	1.859548	1.859548	1.859548	1.859548
P(T<=t) 2-tail		4.66E-07	3.29E-05	3.82E-07	1.725E-07	0.356874	3.6E-08
Critical t 2-tail		2.306006	2.306006	2.306006	2.306006	2.306006	2.306006